University of Alberta

Studies of Immunosuppressive Poxviruses

by

Karen Louise Mossman

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Biochemistry

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For Andrew and Jocelyn

Abstract

Poxviruses are large DNA viruses which replicate within the cytoplasm of a host cell. Poxviruses possess the capacity to encode not only enzymes and factors required for progeny formation, but also virulence factors and host range proteins which allow for circumvention of the host immune system.

Myxoma virus is a *Leporipoxvirus* which causes a rapidly lethal infection known as myxomatosis in the European rabbit. The focus of this thesis was to identify myxoma virus virulence factors and enzymes critical for progeny formation and immune circumvention. Characterization of M-T7, a soluble interferon-γ receptor homolog, showed that M-T7 is a stable protein produced by a strong early promoter, and is efficiently translated and secreted from infected cells. Competition binding and inhibition assays revealed that M-T7 binds rabbit interferon-γ with high affinity (Kd = 1.2 nM), and specifically inhibits the biological effects of rabbit interferon-γ. In contrast, orthopoxvirus homologs were found to bind a variety of interferon-γ ligand species. Targeted disruption of both copies of the M-T7 gene demonstrated that within the European rabbit M-T7 retards inflammatory cell migration into infected tissues and disrupts the communication between lymphocytes within the primary site of infection and secondary immune organs.

Cloning and sequencing of the M-T5 gene identified a novel member of the poxvirus host range superfamily, which defines cellular specificity within its natural host. Disruption of the M-T5 gene from the myxoma virus genome resulted in the rapid inhibition of host and viral protein synthesis in infected rabbit lymphocytes, followed by apoptotic death. Within the European rabbit, M-T5 permits the productive infection of peripheral lymphocytes, facilitating virus dissemination to secondary tissue sites and initiating a cellular reaction within

secondary immune organs. Thus, both M-T7 and M-T5 function as critical myxoma virus virulence factors.

Finally, characterization of the I1L gene product revealed enzymatic activity of a dual specific protein tyrosine phosphatase towards tyrosine and serine phosphorylated substrates. Disruption of the I1L gene resulted in a non-viable virus, indicating that I1L is an essential myxoma virus gene.

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LIST OF ABBREVIATIONS

ADCC	antibody-dependent cell-mediated cytotoxicity
AF-1	interferon-γ accessory factor 1
bp	base pair
cDNA	complementary DNA
CTL	cytotoxic T lymphocyte
DMEM	Dulbecco's minimal essential medium
ds	double stranded
E. coli	Escherichia coli
EDTA	ethylene diamine tetraacetic acid
EEV	extracellular enveloped virus
EGF	epidermal growth factor
eIF2	eukaryotic initiation factor 2
FCS	fetal calf serum
GAS	interferon-γ activated sequence
GST	glutathione S-transferase
IFN	interferon
IFNR	interferon receptor
IL	interleukin
iNOS	inducible nitric oxide synthase
INV	intracellular naked virus
ISGF	interferon stimulated gene factor
ISRE	interferon stimulated response element
JAK	Janus kinase
K_d	dissociation constant
MBP	myelin basic protein
MGF	myxoma growth factor
MHC	major histocompatibility complex
moi	multiplicity of infection
mRNA	messenger RNA
NK	natural killer
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pNPP	p-nitrophenyl phosphate
SDS	sodium dodecyl sulfate
serpin	serine protease inhibitor
SFV	Shope fibroma virus
SH2	src-homology 2
STAT	signal transducer and activator of transcription
TdT	terminal deoxynucleotidyl transferase
TIR	terminal inverted repeat
TNF	tumor necrosis factor
VCP	virus complement control protein
VSV	vesicular stomatitis virus
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactoside

LIST OF VIRUSES

vMyx	wild type myxoma virus (strain Lausanne)
vMyxlac	vMyx (*LacZ*$^+$)
vMyx-I1L$^-$	vMyx with I1L gene disruption (*LacZ*$^+$)
vMyxlac-T7gpt	vMyxlac with disruption of both copies of M-T7 (*LacZ*$^+$*gpt*$^+$)
vMyxT7rev	vMyxlac-T7gpt with reconstitution of both copies of M-T7 (*LacZ*$^-$*gpt*$^-$)
vMyxT5$^-$	vMyx with disruption of both copies of M-T5 (*gpt*$^+$)
vMyxlacT5$^-$	vMyxlac with disruption of both copies of M-T5 (*LacZ*$^+$*gpt*$^+$)
vMyxT5rev	vMyxlacT5$^-$ with reconstitution of both copies of M-T5 (*LacZ*$^-$*gpt*$^-$)

LIST OF VIRUS GENES

M-T2	myxoma virus TNF receptor homolog gene
M-T5	myxoma virus host range superfamily member gene
M-T7	myxoma virus IFN-γ receptor homolog gene
M11L	myxoma virus membrane associated protein
MGF	myxoma virus growth factor gene
Serp1	myxoma virus serine proteinase inhibitor gene
I1L	myxoma virus tyrosine/serine phosphatase gene
H1L	vaccinia virus tyrosine/serine phosphatase gene
M1L	Shope fibroma virus tyrosine/serine phosphatase gene
ST7	Shope fibroma virus IFN-γ receptor homolog gene
SPV-C6L	swinepox virus IFN-γ receptor homolog gene
VV-B8R	vaccinia virus IFN-γ receptor homolog gene
VAR-B8R	variola virus IFN-γ receptor homolog gene
CHO-hr	cowpox virus host range superfamily member gene
VV-B4R	vaccinia virus host range superfamily member gene
VV-K1L	vaccinia virus host range superfamily member gene
VV-C7L	vaccinia virus host range superfamily member gene
VAR-B6R	variola virus host range superfamily member gene
EV-K1L	ectromelia virus host range superfamily member gene
RPV-SPI-1	rabbitpox virus serine proteinase inhibitor and host range superfamily member gene

CHAPTER I

General Introduction

As obligate intracellular parasites, viruses have evolved to both exploit host cell components and encode functions not readily available from their host. By virtue of their ability to replicate and produce viable progeny with a relatively limited genome, viruses are among the most efficient of all life forms. Environmental diversification and host evolution demand constant adaptation by viruses in order to successfully propagate. Both mutation and recombination introduce substantial diversity within the viral genome, especially given the high level of replication in a short time frame. In addition, the capacity to encode factors not required for replication but for host immune evasion facilitates survival. As host organisms develop exceedingly complex defence systems, so must viruses develop effective counteractive mechanisms. Furthermore, viruses must establish an intricate balance between self propagation and host survival in order to prevent their annihilation.

The Poxviruses

The largest of all eukaryotic DNA viruses, poxviruses provide significant insight into the intricate relationship between a virus and its host. Poxviruses infect a wide variety of vertebrate and invertebrate hosts, and are classified into two subfamilies accordingly: the vertebrate poxviruses (Chordopoxvirida) and the insect poxviruses (Entemopoxviridae). The vertebrate poxviruses, which exhibit sufficient similarity to participate in nongenetic reactivation and share a group-specific antigen (47, 169), are further subdivided into eight genera, whereas the insect poxviruses, on which limited information exists, are grouped into three distinct genera. In general, members of the same genera possess similar morphological and biological characteristics.

General Features of Poxviruses

Poxviruses as a whole possess several distinguishing features, the most unique of which is the cytoplasmic localization of viral replication and progeny formation. Poxvirus genomes consist of a single linear, double stranded DNA (dsDNA) molecule with covalently closed hairpin termini and terminal inverted repeat (TIR) regions. Genomes range in length from 130 to 300 kilobase pairs (kbp), and display a high gene density with the ability to encode approximately 200 polypeptides. Each DNA genome is complexed with several proteins, forming a nucleoprotein fiber which is twisted and folded into a biconcave core structure. Nestled in the concavities of the core are two lateral bodies whose function remains elusive. Surrounding the core and lateral bodies is an outer membrane comprised of a lipoprotein bilayer. These large, complex virions, which appear oval or brick-shaped, contain many if not all of the enzymes required for the transcription of polyadenylated, capped and methylated viral early messenger RNA (mRNA). Several excellent and comprehensive reviews on the properties of poxviruses are available (20, 103, 104).

Poxvirus Replication and Progeny Formation

Poxvirus replication is both highly complex and autonomous, consisting of virus entry, three phases of regulated gene expression, DNA replication, virion assembly and progeny dissemination (20, 103, 104, 149). Upon fusion with the cell membrane and entry into the cytoplasm of a host cell, early viral mRNA is transcribed within the viral cores by virion associated proteins, and then extruded from the cores to associate with ribosomes within the cytoplasm. Infectious poxvirus particles contain a complete transcriptional system of enzymes and factors, including RNA polymerase, poly(A) polymerase, RNA methyltransferase, capping enzymes, and DNA topoisomerase (103, 104).

Translation of early viral enzymes allows for both further uncoating of viral cores and DNA replication of viral genomes. DNA replication occurs in discrete areas termed viral factories or B-type inclusions, and is required for late gene expression. Virion morphogenesis follows, producing two forms of infectious mature progeny, the intracellular naked virus (INV) and the extracellular enveloped virus (EEV). While INV appears to be more abundant, release of EEV from infected cells is believed to be crucial for successful virus propagation.

By progressing through distinct and temporally regulated phases, the life cycle of a poxvirus mirrors that of the eukaryotic cell. Progression often correlates with the changing phosphorylation states of regulatory proteins (144), and thus it is not surprising that viruses encode both protein kinases and protein phosphatases (81, 82). Poxvirus virions are known to contain abundant kinase activity (115), and to date two essential vaccinia virus virion-associated kinases have been characterized (8, 85). The protein product of the B1R open reading frame, a 34 kiloDalton (kDa) serine/threonine protein kinase, is required for the synthesis of both DNA and late proteins (8, 86, 124), and is believed to play a role in viral gene transcription, due to its location within the virion. Potential physiological substrates of the B1R kinase include the 40S ribosomal subunits (8), whose state of phosphorylation increases upon infection with vaccinia virus (19). It is speculated that phosphorylation of the ribosomal subunits and modification through binding to the 11 kDa virion phosphoprotein (129) may confer translational specificity for viral mRNA. The major vaccinia virus virion-associated protein kinase is a 50 kDa serine/threonine protein kinase encoded by the F10L open reading frame (85). This kinase is required for the initial stages of virion morphogenesis and, like the B1R kinase, may play a role in early gene transcription due to its location within the virion (85, 150).

Protein phosphatases play a crucial role in counteracting the effects of protein kinases, thus regulating both cellular and viral life cycles. The vaccinia virus VH1 protein, the original and prototypic member of the dual specificity class of phosphatases (56), is packaged into virions and is essential for viral transcription (88). Repression of VH1 expression results in both the hyperphosphorylation of the virion-associated 11 kDa histone-like DNA binding protein, and the inability to direct the transcription of early genes (88). Thus poxvirus encoded kinases and phosphatases parallel their cellular counterparts by influencing the progression of replication and progeny formation.

Changes in protein phosphorylation early in infection correlate with the inhibition of host cell metabolism, a hallmark feature of poxvirus infection (20, 103). Although the mechanism remains obscure, inhibition of host protein synthesis can occur in the absence of viral gene expression, implicating at least one virion associated protein. The 11 kDa virion phosphoprotein has been shown to inhibit protein synthesis *in vitro* by inhibiting the 40S ribosomal subunit/methionyl transfer RNA initiation complex; however, there appears to be no discrimination between host and viral protein synthesis (118, 119). Selective inhibition of host mRNA translation does occur following the synthesis of small, poly(A)-containing RNA molecules by vaccinia virus cores *in vitro* (6, 7), although little is known what effects these molecules have *in vivo*. Several lines of evidence also implicate early viral proteins in the inhibition of host protein synthesis. Studies with cyclohexamide have shown that an irreversible translation block of host and viral mRNA occurs in the absence of early viral protein synthesis (105). In addition, studies with viral proteins implicated in host range determination have shown that restricted growth of a poxvirus in a non-permissive cell line is accompanied by early viral mRNA but not early viral protein synthesis, and host protein synthesis is rapidly shut-off (31, 32, 60, 108).

Orthopoxviruses

The orthopoxvirus genus contains ten species, including vaccinia virus, variola virus, cowpox virus and ectromelia virus (46). Variola virus, a strictly human pathogen, was responsible for world wide pandemics of smallpox, which resulted in the deaths of millions of people. In 1796, Edward Jenner discovered and promoted the use of cowpox as a means of protection from smallpox infection (12). However, laboratory propagation of these viruses led to the observation that material from a typical cowpox lesion was substantially different from that harvested from lesions produced by the smallpox vaccine virus, now known as vaccinia virus (30). Subsequent vaccination with vaccinia virus led to the eradication of smallpox by 1977, culminating in the most successful antimicrobial campaign in medical history (42). Vaccinia virus continues to be used as a vector in engineering recombinant vaccines (106, 146), and is the most widely studied poxvirus.

The origin of vaccinia virus remains obscure, although several hypotheses have been postulated (40). These hypotheses include the derivation from either cowpox virus or variola virus by passage in cows or other animals, the attenuation and alteration of variola virus by continuous passage in human skin, formation of a hybrid between cowpox and variola viruses, and the maintenance in the laboratory of a living representative of a now extinct virus. Since the evolutionary history and natural host(s) of vaccinia virus are unknown, true pathological studies are difficult to perform and have been replaced with intranasal or intracranial inoculations of mice in order to study virulence.

While little is known about the origin of vaccinia virus, much is known about the genome sequence and structure. The vaccinia virus genome consists of 191 kbp, with the potential to encode for 263 genes. Both the vaccinia virus

and variola virus genomes have been completely sequenced (53, 95), allowing for the characterization of numerous open reading frames based on sequence homology. Organization of the vaccinia virus and variola virus genomes is conserved between members of the orthopoxvirus genus and other poxviral genera. Genes encoding proteins essential for viral replication and structure are positioned within the central regions of the genome, while genes critical for pathogenesis and virus-host interactions are located within the genomic termini (20, 148, 152).

In addition to vaccinia virus, ectromelia virus has also been used extensively as an experimental poxvirus in the study of pathogenesis and viral immunology (20, 39, 46). Ectromelia virus, the causative agent of mousepox, induces minimal, nonfatal lesions in genetically resistant strains of mice, while in genetically susceptible strains of mice, death results within one week due to a disseminating infection of the liver and spleen with resultant necrosis. The natural reservoir of ectromelia virus is unknown, although this orthopoxvirus is relatively unique in possessing a very narrow host range, infecting only certain species of mice (20).

Leporipoxviruses

Members of the *Leporipoxvirus* genus include myxoma virus, Shope fibroma virus (SFV), malignant rabbit fibroma virus (a natural recombinant between myxoma virus and SFV), squirrel fibroma virus and hare fibroma virus (96, 97). While all members of this genus can infect *Oryctolagus cuniculus*, the domestic European rabbit, only myxoma virus and SFV have naturally evolved within the rabbit. Myxoma virus and SFV naturally infect species of *Sylvilagus*, one of nine genera within the Leporidae family.

Shope Fibroma Virus

SFV was initially isolated from a subcutaneous fibroma derived from a wild cottontail rabbit, *Sylvilagus floridanus*, which has been recognized as its natural host (139). Infection of the cottontail rabbit with SFV causes large fibromas, primarily on the legs and feet, which appear 7 to 8 days post infection and persist for many months (96). The fibromas exhibit extensive dermal proliferation accompanied by viral replication and the production of infectious progeny, which are mechanically transmitted via arthropod vectors. Similar localized benign fibromas are also evident after SFV infection of the domesticated European rabbit and additional species of *Sylvilagus*. SFV possesses a genome 160 kbp in length, 25 kbp of which comprise the TIRs. The SFV TIRs have been completely sequenced (154, 158), and a composite physical map of the genome has been constructed (26).

Myxoma Virus and Myxomatosis

Myxoma virus was originally discovered in Uruguay in 1896 when imported laboratory rabbits were stricken with an unidentified illness now recognized as myxomatosis (44). The California and South American strains of myxoma virus evolved in *Sylvilagus bachmani* and *Sylvilagus brasiliensis*, respectively, although under natural conditions myxoma virus is able to infect rabbits of the *Sylvilagus*, *Oryctolagus* and *Lepus* genera (44). Myxoma virus resembles SFV in causing the formation of localized benign fibromas upon infection of its natural host. However, in the European rabbit, myxoma virus causes a systemic lethal disease known as myxomatosis (41, 43, 44, 96, 97). Myxomatosis is characterized as a generalized infection with large raised lesions at the primary site of inoculation which become congested and necrotic, extensive viral dissemination culminating in high virus titres in most tissues,

secondary tumors on the nose, ears and eyes accompanied by purulent exudate, and a progressive secondary Gram-negative bacterial infection of the respiratory tract and conjunctiva. Infection with myxoma virus results in >99% mortality, with death occuring due to a combination of tissue damage, immunosuppression, and bacterial infection. Unlike SFV, myxoma virus is able to replicate within the lymphocytes and monocytes of infected European rabbits, which is crucial for the dissemination of virus to peripheral sites (96, 97, 111). While transmission within its natural host is limited to dermal inoculation by arthropod vectors, myxoma virus can also be transmitted to European rabbits through contact infection of ocular and skin lesion discharge containing high levels of infectious virus (41, 44).

Due to the high mortality rate, myxoma virus was deliberately used for the biological control of wild European rabbits in Australia and France in the early 1950's (41, 44, 45). The European rabbit was introduced into Australia in 1859, and within twenty years had become a major pest due to the lack of natural predators. The initial spread of myxoma virus resulted in a high level of mortality, although a rapid co-evolution between virus and host ensued (41, 44). Selection for attenuated viral strains with reduced host mortality and increased periods of transmissibility occurred alongside the selection of rabbits with a greater innate resistance. While myxoma virus is now endemic in Australia, with rabbit populations approaching the levels prior to the initial virus release, the history of myxomatosis in Australia has provided a unique opportunity to study the co-evolution between a virus and its host.

Numerous similarities exist between myxoma virus and SFV, suggesting a close evolutionary relationship. The myxoma virus genome is similar in length to that of SFV (26, 128), and demonstrates conservation of both genomic organization and viral gene sequence (18, 155, 156, 158, 159). Under

conditions of moderate stringency, myxoma virus and SFV DNA demonstrate considerable cross-hybridization (18, 168), and protection of domestic rabbits from myxoma virus results upon prior inoculation with SFV (139).

Myxoma virus provides an excellent model system for studying poxvirus pathogenesis and virus-host interactions for several reasons. First, the evolutionary history of myxoma virus has been well established, allowing for the investigation of viral immune subversion within a biologically relevant context. Secondly, the disease profile of myxoma virus in the European rabbit has been extensively studied, offering a well-defined pathology in an animal suited for experimentation. Finally, field experiments in Australia rendered the collection of both natural attenuated myxoma virus variants and resistant strains of rabbits, which is invaluable in ascertaining the nature of virus virulence in an outbred immunocompetent host.

Host Response to Poxvirus Infection

The complex nature of a poxvirus infection induces an impressive array of host defenses, which rely on both nonspecific and learned (immune) responses. The host response to a poxvirus infection has been extensively reviewed (20, 40, 152), and is only briefly mentioned in the following sections.

Immune Responses

Humoral Response

Specific antiviral antibodies can control virus spread through a variety of mechanisms which include masking receptors for viral attachment, inhibiting viral internalization and/or uncoating, eliciting viral aggregation, enhancing both cytolysis in collaboration with complement and phagocytosis of virally infected cells, and upregulating antibody-dependent cell-mediated cytotoxicity (ADCC).

During a poxviral infection, antibodies to both virions and soluble antigens are observed, although the ability of a particular antiviral antibody to be effective varies substantially. Immune antisera raised against INV is neither able to neutralize virus *in vitro* nor protect an uninfected host by passive transfer *in vivo*, whereas immune antisera to EEV not only neutralizes and protects, but supports ADCC as well (5, 151).

ADCC, in which several different leukocyte populations are capable of lysing virus infected cells precoated with antiviral antibodies, is the major cell mediated method of killing vaccinia virus infected cells during a secondary infection (102, 117). Likewise, ADCC activity is observed well after the initiation of tumor regression in SFV infected rabbits (136), indicating that ADCC is important in the containment and elimination of virus within a secondary infection. In general, the humoral arm of the host immune response appears to be useful for the reduction of the severity of viral disease and the inhibition of secondary viral spread, along with providing immunity to animals upon subsequent challenge with a particular poxvirus.

Cellular Response

While the humoral response functions in controlling a secondary poxvirus infection, the cellular response is important in protecting against a primary poxvirus infection. Cellular immunity is provided by T lymphocytes, which exercise a complex array of antiviral mechanisms both directly, by lysing virally infected cells, and indirectly, by secreting antiviral factors collectively named lymphokines. Both $CD4^+$ and $CD8^+$ T lymphocytes function in the immune response to poxviral infection, and their importance was confirmed in experiments in which increases in morbidity and mortality were observed

following infection of mice with either ectromelia virus or vaccinia virus which were depleted of T-cells using anti-thymocyte antiserum (16, 58).

$CD8^+$ T-cells, also refered to as cytotoxic T lymphocytes (CTLs), recognize surface membrane composition changes in poxvirus infected cells which occur prior to the onset of DNA replication. As a result, cell lysis occurs before progeny formation is complete, thus limiting dissemination to neighbouring cells and peripheral locations (172). Initial studies revealed a correlation between virus clearance and upregulation of CTL activity, with a higher virus dose eliciting a greater CTL response (17). $CD4^+$ T-cells function in the generation of specific anti-viral antibodies and CTLs, while also mediating the recruitment of inflammatory cells to the site of infection. The importance of $CD4^+$ T-cells in a poxvirus infection is demonstrated by the observation that myxoma virus infection causes a dramatic downregulation of surface CD4 in a rabbit $CD4^+$ T-cell line (9).

While both classes of T lymphocytes function in the cellular immune response to poxvirus infection, it appears that the $CD4^+$ response is secondary to that of CTLs. Genetically resistant mice that were depleted of $CD4^+$ T-cells developed an optimum virus-specific CTL response and fully recovered from infection with ectromelia virus, which was lethal in $CD4^-$ $CD8^-$ nude mice (21). Also, mice suffering from mousepox demonstrated detectable levels of virus-specific CTLs 4 days post infection while neutralizing antibody was detectable in the circulation 8 days post infection (40). Furthermore, in adult rabbits infected with SFV, maximum CTL activity is reached when fibroma regression is first detected, while the $CD4^+$ response correlates with the final stages of regression (136). Thus, it appears that CTLs with immunologic specificity for poxvirus-induced antigenic changes in infected cell surface membranes comprise the backbone of the host response to poxvirus infection.

Nonspecific Responses

Prior to the onset of the learned responses of humoral and cellular immunity, nonspecific antiviral responses are unleashed by an infected host. These nonspecific responses include natural killer (NK) cell activity, complement cascade, inflammation, apoptosis and lymphokine production.

NK Cell Activity

The discovery of NK cells was, in part, due to their ability to kill virally infected cells. Unlike CTL lysis, NK cell lysis does not require specific viral antigens, and is thus part of the natural rather than specific immunity. It is believed that NK cells serve to lyse virally infected cells until antigen-specific CTLs are formed, and, as a result, individuals who lack NK cells are more susceptible to severe viral infections (1). Aside from spontaneously killing virally infected cells, NK cells secrete antiviral cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor α (TNFα), and are the predominant cellular mediators of ADCC. In mice infected with vaccinia virus, NK cell depletion results in an increase in both the size of the lesion and virus titre in the spleen and liver (164), demonstrating the requirement of NK cell activity during poxvirus infection.

Complement Cascade

The complement cascade consists of functionally linked serum proteins that interact with one another in a highly regulated manner to provide many of the effector functions of the humoral defense system. Two pathways exist, the antibody dependent classical pathway and the antibody independent alternative pathway. Activation of complement components results in the neutralization and lysis of virions, an increased rate of phagocytosis and lysis of

virus infected cells, and enhancement of the inflammatory response. Vaccinia virus encodes a 35 kDa protein, refered to as VCP (virus complement control protein), which is homologous to the human C4b binding protein (67, 77, 78). VCP inhibits both the classical and alternative pathways, and deletion of the gene encoding VCP results in vaccinia virus attenuation in mice.

Inflammatory Response

Inflammation is characterized as a localized protective response against injury and tissue damage, which serves to destroy or sequester both the injurious agent and the injured tissue. Prior to the development of an immune response, inflammation is detected in lesions induced by a variety of poxviruses, including vaccinia virus and SFV (20). Inflammatory cells may limit viral spread by the production of oxygen dependent and independent antimicrobial products, phagocytosis, or the secretion of antiviral lymphokines such as interferon. The exact role played by inflammatory cells in poxvirus clearance remains obscure; the inflammatory response to a primary poxvirus infection is insufficient to dissolve the infection, requiring instead the cooperation of the learned immune response (20).

Studies of chicken embryos, which lack mature T and B cells, infected with cowpox virus resulted in the discovery of the first poxvirus serine protease inhibitor (serpin). The 38 kDa cowpox virus protein, refered to as SPI-2 or crmA, functions to inhibit the inflammatory response, and when deleted from the cowpox virus genome induces the formation of white pocks, characterized by the massive infiltration of inflammatory cells, in the place of red hemorrhagic pocks seen with wildtype virus (114, 121). There now exists a family of poxvirus serpins, which function to interfere with both clotting and wound regression (153).

Apoptosis

Apoptosis, or programmed cell death, is a naturally occuring process of cell suicide which functions in the regulation of cell populations and elimination of unwanted cells. Apoptosis serves as a protective mechanism against viral infection since apoptotic cells, along with associated intracellular virus, are rapidly ingested by phagocytic cells, preventing viral spread. Poxviruses have developed mechanisms to subvert the host apoptotic response by encoding a number of genes whose products either delay or prevent programmed cell death in specific cell types. Proteins encoded by the cowpox virus SPI-2 and CHOhr genes, and the vaccinia virus E3L gene, function to inhibit apoptosis induced upon viral infection (64, 83, 147). In addition, these three proteins function in the determination of host range. Myxoma virus also encodes three proteins, M-T2, M-T5 and M11L, which are involved in suppressing the host apoptotic response (91 & Chapter VI, this thesis).

Interferon

IFNs were discovered in 1957 based on their ability to interfere with viral multiplication and render cells immune to viral infection (65). Soon after their discovery, IFNs were shown to protect rabbits from intradermal infection by vaccinia virus (66). Additionally, supernatants from mouse cells infected with vaccinia virus at a low, but not a high, multiplicity of infection (moi) provided protection to neighbouring cells, demonstrating that the local production of IFN protects uninfected neighbouring cells from poxvirus infection (52). Studies of animals infected with either ectromelia virus, vaccinia virus or SFV demonstrated a dramatic induction of IFN in the serum, spleen, liver, lymph nodes and primary lesions of the animals, with enhanced IFN levels correlating with a loss of infectious SFV in lesions in rabbits (20).

Three major classes of IFNs, IFN-α, IFN-β and IFN-γ, appear to be present in all mammalian species. In addition to their antiviral activity, IFNs are recognized as pleiotropic cytokines that provide defense against microbes, tumors and antigens, as well as playing an important role in the regulation of a wide range of immune and inflammatory responses (27, 68, 120, 137, 162).

Type I IFNs: IFN-α and IFN-β

The type I IFNs include IFN-α and IFN-β, known as leukocyte and fibroblast IFN, respectively (for a comprehensive review, refer to reference 160). IFN-α constitutes a large family of at least 23 structurally related proteins, which possess 94% homology at the nucleotide level and range in molecular mass from 16 to 27 kDa. IFN-β exists as a single 20 kDa species, and demonstrates 25% amino acid identity with members of the IFN-α family. Genes encoding the type I IFNs are normally silent, but are rapidly activated upon viral infection, with the major stimulus being dsRNA. In the early stages of infection, macrophages represent the major source of type I IFN, although almost all cell types are known to express both IFN-α and IFN-β.

Type I IFNs function primarily as antiviral cytokines, although they also demonstrate antiproliferative and antitumor activity, modulate cellular differentiation, and stimulate both expression of MHC I molecules and cytotoxic activity of lymphocytes and phagocytes. While all IFN-α subtypes show antiviral and antiproliferative properties, considerable variation is seen in their specific activities, and although IFNs in general display species specificity, some human IFN-α species are active on murine and bovine cells. Due to their biological properties, type I IFNs are currently being used to treat patients with certain virus infections (48).

Both IFN-α and IFN-β bind to the same type I IFN receptor, which consists of at least two distinct polypeptides of 110 and 102 kDa, and is found on most cell types in low abundance. Coexpression of the two cloned type I IFN receptor components invokes antiviral activity but not antiproliferative responses, indicating that these two receptor proteins are insufficient to induce a complete biological response, implicating additional components (51). It is also believed that IFN-α and IFN-β either interact with a different accessory receptor component or interact with the same accessory receptor component in a different manner, since these two type I IFNs exhibit different biological responses to similar stimuli. In addition to the cellular receptor complex, a soluble form of the 102 kDa polypeptide exists, which is a potent blocker of all type I IFNs (109). The soluble receptor is encoded by its own mRNA, and thus is subjected to independent regulation in contrast to being produced by proteolysis of the cell surface receptor.

Type II IFN: IFN-γ

IFN-γ, the lone type II IFN, is distinguished from type I IFNs by a number of factors: (1) a single IFN-γ gene exists, which displays little sequence similarity to the IFN-α and IFN-β genes and has a different chromosomal location, (2) the IFN-γ gene has four exons and three introns while the type I IFN genes are intronless, (3) IFN-γ is antigenically distinct, (4) IFN-γ is inactivated at low pH, whereas the type I IFNs are not, (5) specific cell types produce IFN-γ, (6) IFN-γ exhibits strict species specificity, and (7) the main function of IFN-γ is to serve as an immunoregulator as opposed to an antiviral cytokine (37, 69).

T cells and NK cells account for the majority of IFN-γ production, although other cells have been reported to express IFN-γ, presumably at basal levels with little or no biological importance (170). While many different stimuli are capable

of eliciting the production of IFN-γ, both Th1 $CD4^+$ and $CD8^+$ T cells produce IFN-γ primarily in response to antigens in the context of MHC II and MHC I, respectively, while interleukins (ILs) 2 and 12 serve as the major inducers of IFN-γ in NK cells (170). IFN-γ gene expression, which is closely linked to T cell activation, is characterized by rapid mRNA induction and is subjected to regulation at the transcriptional level as well as through DNA methylation and mRNA stability.

The human IFN-γ protein precursor contains 166 amino acids, which includes a signal sequence of 23 amino acids. The predicted molecular weight of the secreted IFN-γ monomer is 17 kDa, however, three distinct forms of secreted IFNγ monomer have been observed to migrate at 17 kDa, 20 kDa and 25 kDa upon SDS polyacrylamide gel electrophoresis (SDS-PAGE) (72). This heterogeneity is likely due to variable amounts of carbohydrate at two N-linked glycosylation sites (125). While glycosylation of the ligand is not required for binding to the IFN-γ receptor (IFN-γR) and subsequent downstream signalling, it does affect the half-life of the mature ligand in the circulation and possibly within tissue sites as well.

The human IFN-γR, devoid of an N-terminal 17 amino acid signal sequence, consists of a 228 amino acid extracellular domain, a 23 amino acid transmembrane domain and a 221 amino acid intracellular domain (37). The IFN-γR acquires N-linked sugars during transport from the endoplasmic reticulum through the Golgi, and although only fully glycosylated receptors are expressed on the cell surface, glycosylation has been found to be nonessential for binding of the receptor to its ligand. Cell specific differences in glycosylation probably accounts for the heterogeneity in reported molecular weights (approximately 80-95 kDa) for the human IFN-γR. Since the IFN-γR sequence

contains no obvious identity to other receptors, it has been assigned to the new type II cytokine receptor family (13, 14, 74, 101).

IFN-γRs are expressed on virtually all cell types, at levels varying between 10^2 and 10^4 receptors per cell (161). The only exceptions appear to be erythrocytes, which do not express IFN-γRs, and T lymphocytes, which are either devoid of IFN-γRs, or express extremely low levels. The receptor has a single, high affinity binding site for IFN-γ, with reported dissociation constant (K_d) values ranging from 10^{-10} to 10^{-11} M (2). Almost the entire extracellular domain is required for ligand binding, and formation of four consecutive disulfide bonds within this domain is critical for proper folding and biological activity (50, 143). Although the intracellular domain is devoid of catalytic activity, a transmembrane proximal region is required for receptor mediated ligand internalization and degradation, and induction of a biological response, while a C-terminal region is required exclusively for induction of a biological response (36).

Soluble forms of the extracellular ligand binding domain of human IFN-γ exist both in nature and have been engineered in the laboratory (110). Soluble IFN-γRs are active, stable proteins which bind IFN-γ with high affinity (K_d 10^{-9} M), and effectively sequester the ligand away from the membrane bound receptor. Recombinant soluble mouse IFN-γR both neutralizes the protective effects of treatment with murine IFN-γ and inhibits endogenously produced IFN-γ (112) . Due to their IFN-γ antagonistic activities, soluble IFN-γRs are being actively pursued as potential therapeutic agents.

Crystal structure studies of both IFN-γ and a complex between IFN-γ and its soluble receptor have provided much information on IFN-γ ligand-receptor interaction (33, 130, 163). X-ray diffraction studies of crystallized human and rabbit IFN-γ revealed six α-helices held together by short nonhelical regions.

Biologically active IFN-γ functions as a noncovalent homodimer, and upon homodimerization, the α-helices become intertwined in an antiparallel fashion, leading to multiple interactions along the molecular faces. Earlier studies suggested that the N-terminal region formed specific interactions with the receptor, while the C-terminal polycationic region formed nonspecific salt bridges, with dimerization of IFNγ allowing one ligand homodimer to bind two receptor molecules (55). While crystallographic data confirmed that binding of IFN-γ to its receptor induces receptor dimerization, the two IFN-γRs do not interact with each other. The ligand binding portion of the IFN-γR folds into two domains, both of which adopt a fibronectin III domain topology, a feature found in several receptor families (14, 116). Residues from both receptor domains interact with the IFN-γ homodimer, and differences between the human and murine sequences in these areas are sufficient to explain the species specific interaction between IFN-γ and its receptor (163).

When human IFN-γR cDNAs are expressed in murine cells, the receptor is capable of binding and internalizing human IFN-γ with kinetics indistinguishable from their natural counterparts on human cells, however, induction of a functional response is not detectable (3). Instead, an additional receptor component, refered to as the IFN-γ accessory factor (AF-1), is required to form a biologically active IFN-γR complex (142). Induction of a biological response requires the IFN-γ ligand and the extracellular domains of the IFN-γR and AF-1 to be of the same species, whereas the cytoplasmic portions of the two receptor components do not display species specificity (76).

IFN Signal Transduction

IFN signal transduction cascades initiate upon binding of IFNs to their respective cellular receptor complexes. As with other members of the cytokine

receptor superfamily, the *Janus* (JAK) kinases couple ligand binding to the tyrosine phosphorylation of signalling proteins, the signal transducers and activators of transcription (STATs) (24, 62, 63). Even though the type I and II IFN receptor complexes are distinct, overlapping sets of genes are induced by both IFN types, and common elements are found in their signalling pathways (69). The 110 kDa and 102 kDa type I IFN receptors associate with Tyk2 and Jak1, respectively, which tyrosine phosphorylate Stat1 and Stat2. Phosphorylated Stat1 and Stat2 complex to form IFN-stimulated gene factor 3 α (ISGF3α) which migrates to the nucleus, complexes with the DNA-binding ISGF3γ subunit, and activates genes which contain an IFN-stimulated response element (ISRE).

A current model of the IFN-γR complex suggests that the dimerization of two IFN-γR chains upon binding of an IFN-γ homodimer allows for the subsequent association of two AF-1 chains, with the extracellular domains of both the IFN-γR and AF-1 contacting the IFN-γ ligand (76). The IFN-γR and AF-1 chains associate with Jak1 and Jak2, respectively, and ligand-induced receptor aggregation is proposed to aggregate the associated JAKs and allow their activation by transphosphorylation. Activated Jak1 and Jak2 subsequently tyrosine phosphorylate two Stat1 molecules, which then homodimerize through their carboxy-terminal src-homology 2 (SH2) domains. Homodimerization triggers movement to the nucleus and binding to IFN-γ activated sequence (GAS) motifs in IFN-γ stimulated gene promoters. Genes which are stimulated by both type I and type II IFNs often contain both ISRE and GAS motifs within their promoter regions. Furthermore, both IFN-α and IFN-γ have been shown to upregulate the expression of Stat1 and Stat2, enhancing the overall IFN response (79).

Antiviral Mechanisms of Interferons

While IFNs themselves do not directly inhibit viral replication, they instead stimulate a set of effector proteins which induce the antiviral state. Two classic antiviral pathways rely on the enzymatic activities of two different IFN-stimulated gene products, both of which are stimulated primarily by IFN-α and IFN-β, but also by IFN-γ (69). PKR, a dsRNA dependent protein kinase, and 2-5 A synthetase are both induced by IFNs. These enzymes become activated upon binding to viral dsRNA, which accumulates during viral infection. Activation of 2-5 A synthetase by dsRNA catalyzes the linkage of adenosines via 2'-5' phosphodiester bonds into oligomers of adenosine designated 2,5-A, which in turn activate an endoribonuclease, endoRNaseL, resulting in the efficient degradation of RNA (73). Activated PKR undergoes autophosphorylation and the phosphorylated PKR then in turn phosphorylates the translation initiation factor eIF-2α. Phosphorylated eIF-2α is incapable of binding to its co-factor eIF-2β, and thus translational initiation of mRNA becomes blocked (38). PKR has also recently been shown to phosphorylate IκB, the inhibitor of transcription factor NFκB, resulting in the transcriptional activation of NFκB-dependent genes such as IFN-β (167). In addition, PKR has been implicated in affecting the signal transduction pathways of cytokines including IFN-γ (167), and has been shown to induce apoptosis (83).

Although IFN-γ is not predominantly an antiviral cytokine, it does affect viral replication indirectly by serving as a potent immunomodulator. One such important aspect is its ability to serve as a potent macrophage activation factor (133, 135). IFNγ promotes the elaboration of macrophage derived cytocidal compounds such as TNFα, Il-1β and reactive nitrogen intermediates, leading to increased intracellular killing of pathogens including viruses (28, 84). An important feature of IFNγ is the production of an inducible form of nitric oxide

synthase (iNOS) in macrophages (71). iNOS converts a guanidino nitrogen of L-arginine into nitric oxide, a toxic compound shown to enhance cytotoxicity of macrophages as well as inhibit viral DNA synthesis, perhaps by inactivating a key DNA replication enzyme, ribonucleotide reductase (100). IFNγ can also enhance macrophage cytotoxicity in an antibody dependent fashion, by increasing the expression of high affinity Fc receptors on monocytes and macrophages, leading to increased ADCC activity (34).

TNFα and Il-1β, when secreted by IFNγ stimulated macrophages, function not only as cytocidal compounds, but as critical pro-inflammatory cytokines as well (122). TNFα in particular has the ability to increase expression of the adhesion molecules ICAM-1 and ELAM-1 on endothelial cells, while IFNγ increases the expression of ICAM-1 and MHC I on a variety of susceptible cells (29). These adhesion molecules aid in T-cell adhesion and T-cell recruitment during inflammation, stimulating the migration of inflammatory cells to the point of viral infection. IFNγ activation of phagocyte receptors, such as urokinase receptors, also promotes cell-cell interaction, migration of phagocytes through connective tissue, adhesion to the extracellular matrix and chemotaxis (80). Thus, IFNγ plays an important role in responding to viral infections via both the early inflammatory process as well as later events that engender acquired cellular immunity.

Expression of both MHC class I and II glycoproteins are also induced by IFNγ, in contrast to IFNα/β which upregulates only MHC I expression (10). Cells that normally express MHC I molecules generally respond to IFNγ with an enhancement of cell surface levels, while cells that normally do not express MHC II molecules become stimulated to induce transcription of MHC II genes, subsequently allowing for the presentation of exogenous viral antigens to $CD4^+$ T cells (11). Increased levels of MHC glycoproteins lead to enhanced viral

antigen presentation, allowing for more effective clearing of virally infected cells. Finally, IFNγ has been shown to induce secretion of the ligand binding domain of the low density lipoprotein receptor (49). This soluble receptor interferes with viral assembly or viral budding of certain enveloped viruses, although its role against non-enveloped viruses remains to be clarified. Thus IFNγ, though classified primarily as an immunomodulator, has profound and diverse effects on the multiplication and spread of viruses.

IFN-γ and Poxviruses

IFN-γ is paramount in the host defense against poxvirus infection. IFN-γ completely restricts replication of vaccinia virus in macrophages, and confers antiviral protection to neighbouring cells by releasing nitric oxide into the culture supernatant (71). In cultured macrophages, the presence of an iNOS inhibitor reverses the IFN-γ induced inhibition of ectromelia virus and vaccinia virus replication, while in genetically resistant mice, a resolving ectromelia virus infection is converted into fulminant mousepox upon the introduction of an iNOS inhibitor (71). Recent studies have demonstrated that IFN-γ induced nitric oxide synthesis inhibits vaccinia virus late protein synthesis, DNA replication, and viral particle formation (57, 100). The abortive infection in iNOS-expressing macrophages limits virus spread, while nitric oxide production at the foci of infection retards viral replication prior to the establishment of specific antiviral CTLs and antibodies.

Early studies with anti-thymocyte serum demonstrated the critical role played by cellular immunity in the clearance of poxvirus infections (16, 58). More recently, the importance of T cells has been clarified: T cells are required for their ability to produce IFN-γ. Activated $CD8^+$ CTLs are predominant early in response to vaccinia virus infection, and are the major producers of IFN-γ at the

height of the antiviral cellular immune response (127). Athymic nude mice and sublethally irradiated euthymic mice resolve an otherwise lethal vaccinia virus infection when infected with a recombinant vaccinia virus expressing murine IFN-γ (75). Furthermore, depletion of IFN-γ with specific monoclonal antibodies renders mice susceptible to lethal infection with vaccinia virus and ectromelia virus, despite normal levels of CTLs and NK cell cytolytic activity (70, 126). Similar results are seen in IFN-γR knockout mice, which show increased susceptibility and mortality to vaccinia virus due to a severely defective early defence despite normal CTL and T helper cell responses (61). It has been suggested that the role of CTLs in a poxvirus infection is not to directly lyse infected cells, but to target antiviral cytokines, predominantly IFN-γ, to the site of infection (123). It is also interesting to speculate whether IFN-γ is important because it is a part of the cellular immune response, or whether cellular immunity is critical because of the activities of IFN-γ.

Poxviral Anti-IFN Strategies

The ability of poxviruses to overcome the antiviral effects of IFN was first observed when vaccinia virus demonstrated resistance to this antiviral agent in a number of cell systems (171). Co-infection studies with vaccinia virus demonstrated the ability of this poxvirus to rescue vesicular stomatitis virus and encephalomyocarditis virus from the inhibitory effects of IFN (165, 166). The resistance of vaccinia virus to IFN as well as the ability of this poxvirus to rescue other viruses was found to correlate with the inhibition of the IFN-mediated enzyme activities of PKR and 2-5 A synthetase (35, 113). This IFN resistance of vaccinia virus is mediated through the elaboration of two early viral gene products, E3L and K3L (22, 25). The vaccinia virus E3L gene product is a dsRNA binding protein which effectively competes with PKR in binding dsRNA,

preventing the activation of this antiviral enzyme. The vaccinia virus K3L gene product is a homolog of eIF2α, and acts as a decoy of eIF2α to prevent its phosphorylation by PKR. Recent studies with E3L$^-$ and K3L$^-$ mutant vaccinia viruses suggest that while E3L directly inhibits PKR-induced antiviral activity, K3L indirectly effects the IFN-resistant phenotype of vaccinia virus, and may instead primarily function in the optimal translation of viral mRNA species and be required for the optimal expression of viral proteins which allow vaccinia virus to evade direct antiviral pathways (15).

In addition to inhibiting antiviral pathways induced by IFNs, poxviruses have developed a unique strategy to intercept IFN ligands prior to receptor binding and initiation of antiviral pathways. The first demonstration of this strategy was revealed upon the characterization of the myxoma virus M-T7 protein, the most abundantly secreted protein from myxoma virus infected cells (159). M-T7, so designated because it is the seventh open reading frame from the myxoma virus genome termini, is present in two copies as it resides within the TIR and encodes a 37 kDa, 263 amino acid protein with significant homology to the ligand binding domain of the mammalian IFN-γRs. M-T7 was found to bind rabbit IFN-γ and abrogate the antiviral state induced by rabbit IFN-γ in a rabbit cell line, effectively serving as a soluble IFN-γR homolog (159). Subsequent studies have shown that M-T7 demonstrates species specificity for rabbit IFN-γ and is a critical virulence factor in the establishment of myxomatosis (this thesis).

Soluble IFN-γR homologs have since been discovered in a number of other poxviruses (107), although their biological relevance remains to be determined due to the lack of appropriate experimental systems. The T7 open reading frame of the related leporipoxvirus, SFV, encodes a 265 residue soluble IFNγR homolog, designated S-T7 (158, 159). Vaccinia virus strains

Copenhagen and Western Reserve encode related 272 amino acid homologs from their respective B8R open reading frames (53, 59), and to date all strains of vaccinia virus tested produce a soluble protein capable of binding IFN-γ (4). An ectromelia virus IFN-γR homolog was discovered due to the homology between ectromelia virus and vaccinia virus genomes, revealing an open reading frame encoding a 266 residue protein, whose location within the ectromelia virus genome remains to be determined (this thesis). Two strains of the smallpox agent, variola virus (Bangladesh 1975 and India 1967), have now been completely sequenced and found to encode related soluble IFNγRs, designated B8R and B9R, respectively, comprising 266 amino acids of which 265 are identical (93, 138). Within the suipoxvirus genus, swinepox encodes a 274 amino acid IFNγR homolog from the C6L open reading frame (94). In addition, cowpox, rabbitpox, camelpox, buffalopox, elephantpox and tanapox viruses were shown to secrete IFN-γ binding proteins from infected cells (4 & this thesis). Due to the importance of IFN-γ in controlling poxvirus infections, it is entirely possible that all poxviruses possess a soluble IFN-γR homolog.

Following the discovery of poxviral proteins that intercept IFN-γ prior to receptor engagement, vaccinia virus was found to encode a soluble protein from the B18R open reading frame which binds and inhibits type I IFNs (23, 145). Interestingly, one group found no homology between B18R and the mammalian IFNα/βR, placing B18R within the immunoglobulin superfamily as opposed to the class II cytokine receptor family (145), while a different group identified B18R based on significant regions of homology with the type I IFN receptors, members of the class II cytokine receptor family (23).

Myxoma Virus Virulence Factors

Aside from M-T7 (characterized in this thesis), myxoma virus elaborates a number of virulence factors which collectively conspire to subvert the host immune system and cause a lethal myxomatosis disease syndrome. The myxoma virus virulence factors include M-T5 (this thesis), MGF, M11L, M-T2 and SERP1 (described below).

Myxoma Virus Growth Factor

The myxoma virus growth factor, MGF, belongs to a family of poxvirus proteins with significant homology to epidermal growth factor (EGF) and transforming growth factor-α (99). MGF is composed of 85 amino acids, and contains 12 of 13 residues that are conserved among members of the EGF family, including all six cysteine residues shown to be critical for proper folding and biological activity (155). Studies with a synthetic peptide of MGF, spanning the six cysteine residues implicated in EGF receptor binding, demonstrated that MGF is capable of binding to the EGF receptor, and contains biological activity (87). Deletion analysis of the MGF gene in myxoma virus demonstrated that MGF is an important virulence factor (111). While all clinical stages of myxomatosis were observed in rabbits infected with an MGF$^-$ myxoma virus, the symptoms were moderated and 75% of infected rabbits fully recovered. It has been suggested that poxviral growth factors such as MGF may function to either stimulate the metabolism of neighbouring uninfected cells thus facilitating virus replication, or utilize the EGF receptor as a means of infection (99).

M11L Gene Product

In the construction of an MGF$^-$ myxoma virus, the 3' end of the M11L gene encoding its carboxy terminus, which overlaps the amino terminus of MGF

by six residues, was inadvertently removed (111). Infection of rabbits with this MGF$^-$M11L$^-$ myxoma virus resulted in the development of a single, benign, localized nodule reminiscent of a SFV-induced tumor at the site of inoculation, which completely regressed within two weeks. Subsequent studies with an M11L$^-$ myxoma virus resulted in rabbits which developed primary and secondary tumors larger and more circumscribed than those of wild type myxoma virus, and which demonstrated only mild symptoms of myxomatosis, followed by complete recovery (111). While the function of M11L remains to be determined, it has been shown to be produced at early times, and persists as a 166 amino acid membrane-spanning molecule (54). An insight into the biological importance of M11L was uncovered, however, when rabbit lymphocytes infected with an M11L$^-$ myxoma virus underwent an abortive apoptosis response (91).

M-T2: TNF Receptor Homolog

The first examples of a virally encoded homolog of a cellular cytokine receptor were the TNF receptor homologs encoded by SFV and myxoma virus, designated S-T2 and M-T2, respectively (140, 156). A family of poxvirus TNF receptor homologs has since been identified (141). The 55 kDa secreted M-T2 protein was shown to be an important virulence factor when the majority of rabbits infected with an M-T2$^-$ myxoma virus demonstrated the ability to mount an effective immune response resulting in the recovery of >60% of infected rabbits (156). Secreted M-T2 was subsequently shown to specifically bind and inhibit rabbit TNFα with an affinity comparable to cellular TNF receptors (131). Aside from preventing the cytolysis of virally infected cells by TNFα, M-T2 also serves, in conjunction with M11L and M-T5 (this thesis), to inhibit the apoptotic death of infected rabbit lymphocytes (91). Intracellular M-T2 was shown to be

responsible for the prevention of an abortive apoptotic response in infected cells, and different domains of M-T2 were found to be responsible for implementing the two distinct anti-immune properties of this virus immunomodulator (132, 134).

SERP1

Myxoma virus encodes a 55 kDa serine protease inhibitor, SERP1, which functions to interfere with the inflammatory response initiated during viral infection (92, 157). Mutation of SERP1 within the myxoma virus genome results in a significant attenuation of the virus in European rabbits (92). Interestingly, in SFV, the related leporipoxvirus which causes only a localized infection in the European rabbit, the homologous open reading frame is fragmented and disrupted. Although numerous poxviruses encode serpin proteins (153), SERP1 bears no significant homology to and has no functional relationship with any poxviral serpins. SERP1 is the first and only poxviral serpin shown to bind and inhibit the proteolytic activities of several human proteinases *in vitro* (89), and it has been suggested that SERP1 may function to regulate cytokine activation by proteolytic cleavage (98). While the *in vivo* target of SERP1 has not been identified, the anti-inflammatory properties of SERP-1 were demonstrated in a rabbit atherosclerosis model. Purified SERP1 infused into the arterial wall during balloon angioplasty inhibited the subsequent formation of an atherosclerotic plaque (90).

Thesis Objectives

The purpose of this thesis is to increase the body of knowledge concerning poxvirus pathogenesis and the interaction of poxviruses within their host. Since myxoma virus provides an elegant system in which to study

virulence and virus-host interactions, the majority of work in this thesis deals with the characterization of specific myxoma virus proteins involved in viral replication and viral virulence:

(1) Studies of the myxoma virus M-T7 protein. Preliminary studies with the myxoma virus M-T7 protein identified it as a soluble homolog of the IFN-γR. The promoter region of M-T7 was studied, along with the kinetics of expression and secretion. As well, quantitation of M-T7 protein from infected cells was monitored, in order to determine if biologically relevant amounts of this protein are produced during a viral infection. Mammalian IFN-γRs are known to interact with IFN-γ with high affinity in a species specific manner, and thus a series of experiments were performed in order to determine whether M-T7 also interacts with high affinity for its ligand in a species specific fashion. Finally, by disrupting both copies of the M-T7 gene, studies were undertaken to assess the role this protein plays in the pathogenesis of myxoma virus within the European rabbit.

(2) Studies of the species specificity of M-T7 homologs. While the South American rabbit has been established as the natural host of myxoma virus, the natural hosts of most poxviruses remain obscure. Since all poxviruses tested to date possess an IFN-γR homolog, the species specificity studies of M-T7 were extended to include the IFN-γR homologs of vaccinia virus and ectromelia virus, two orthopoxviruses whose evolutionary histories are unknown. While the vaccinia virus IFN-γR homolog had been identified as the protein product from the B8R open reading frame, the ectromelia virus homolog remained to be cloned and sequenced. Upon characterization of the ectromelia virus IFN-γR homolog, the interaction of the two orthopoxvirus proteins with a variety of species of the IFN-γ ligand were examined and compared with the ligand specificity of M-T7.

(3) Studies of the myxoma virus M-T5 protein. The myxoma virus genome is organized with genes encoding proteins required for replication clustered within the center of the genome while genes encoding for virulence factors reside within the termini of the genome. Within the myxoma virus termini, several open reading frames with potential to encode for virulence factors remain to be characterized. Thus, studies were undertaken to sequence, clone and characterize the M-T5 open reading frame, which exists as two copies within the myxoma virus TIR, and to determine whether the M-T5 protein functions as a virulence factor within the European rabbit.

(4) Studies of the myxoma virus protein tyrosine phosphatase. Sequence analysis of fragments of the SFV genome had revealed the presence of an open reading frame encoding for a protein with homology to protein tyrosine phosphatases. Due to the high degree of homology between SFV and myxoma virus, experiments were designed in order to locate, clone and sequence the myxoma virus protein tyrosine phosphatase gene. The substrate specificities of the myxoma virus phosphatase, along with an inactivated mutant and the vaccinia virus phosphatase, VH1, were investigated.

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CHAPTER II

Materials and Methods

Viruses and Cells.

Myxoma virus (strain Lausanne), vMyxlac, a myxoma virus derivative containing the *Eschericia coli (E. coli)* lacZ marker gene inserted at an innocuous site between the myxoma growth factor and M9 genes (13), SFV, vaccinia virus (strains WR and IHDW), cowpox virus and rabbitpox virus were cultured in primate BGMK cells, in Dulbecco's minimal essential medium (DMEM) supplemented with 10% newborn calf serum. Ectromelia virus (Moscow strain) was maintained in primate BSC-1 cells in Eagle's minimal essential medium plus 10% fetal calf serum (FCS). Vesicular stomatitis virus (VSV) was propagated in Vero cells in DMEM plus 10% FCS. Mouse L929 cells were maintained in Iscove's modified Dulbecco's medium supplemented with 10% FCS. Rabbit kidney RK13 and rabbit $CD4^+$ T-lymphocyte RL5 (8) cells were maintained in DMEM and RPMI 1640, respectively, each supplemented with 10% FCS. LSTRA cells, used as a source for the tyrosine kinase $p56^{lck}$, were maintained in RPMI 1640 containing 15% FCS and 50μM β-mercaptoethanol. Cowpox and rabbitpox viruses, and VSV were generous gifts of R. Moyer and D. Tovell, respectively. BSC-1, L929 and RK13 cells were obtained from the American Type Culture Collection, whereas RL5 cells were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. BGMK, LSTRA and Vero cells were generous gifts of S. Dales, B. Sefton, and S. Rice, respectively.

Cloning and Sequencing.

The SFV BamHI-M fragment (2, 23) was identified by random sequencing to encode an open reading frame containing homology to the signature sequence motif of tyrosine phosphatases, and was subcloned into pBluescript (Stratagene) in both orientations. Nested deletions with

exonuclease III and exonuclease VII (7) were constructed following cleavage by SstI and BamHI. Sequencing data were compiled to obtain the entire 2.7 kbp SFV BamHI-M fragment sequence. To amplify the SFV open reading frame, M1L, that encoded the putative viral phosphatase, polymerase chain reaction (PCR) was used. The 5' primer incorporated a unique XhoI restriction site upstream of the initiator codon (5'-GGCTCGAGAATGGATAAAAAAAGTC-3'); the 3' primer a BamHI site immediately downstream of the terminator codon (5'-CCGGATCCTTTATGTTTCGTTGATG-3').

To identify the myxoma virus phosphatase gene, genomic vMyxlac DNA was subjected to PCR analysis using the SFV MIL 5' and 3' primers. The amplified 537 bp myxoma fragment was radiolabelled to make probe, by end-labeling with T4 polynucleotide kinase, and used in Southern blot analysis of myxoma DNA digested with BamHI to identify the genomic BamHI clone. The myxoma phosphatase homologue of SFV M1L mapped to the 6.4kb BamHI-I fragment (14), and was designated I1L. This genomic myxoma fragment was subsequently cloned into pBluescript, and sequencing was performed with the SFV M1L 5' and 3' primers, plus four additional primers; these 6 primers were used to obtain the entire myxoma I1L open reading frame, along with 200 bp of both upstream and downstream sequence, in both orientations.

Sequencing of the myxoma virus M-T5 gene was performed using pBamK (described below) as template along with a total of 10 primers directed against both strands of the gene. Initial primers were based on sequences derived from the homologous T5 open reading frame of SFV (20, 22). The ectromelia virus IFN-γ binding gene was localized to a 4.5kb BamHI fragment by Southern blotting using a PCR product of the vaccinia virus B8R gene as probe.

Clone EV-B93 was isolated by size from a shotgun library of ectromelia virus BamHI fragments in pBluescript.

Sequencing of all myxoma virus and SFV DNA was performed on an ABI 373 DNA Sequencer with Taq cycling, to greater than five fold redundancy, whereas sequencing of clone EV-B93 was accomplished using Sequenase II (Amersham) with dsDNA template and custom primers. Sequenced fragments were compiled using GelAssemble (Genetics Computer Group, WI). Sequences of the SFV BamHI-M fragment, the myxoma virus I1L gene, the myxoma virus M-T5 gene, and the ectromelia virus IFN-γ binding gene were submitted to GenBank and assigned accession numbers L32180, L31960, U43201 and U19584, respectively. Additional DNA and peptide sequences were obtained from the PIR and GenBank databases. Sequences were compared and aligned using Bestfit and PileUp, respectively (Genetics Computer Group, WI).

Plasmids.

The glutathione S-transferase (GST) gene fusion system (Pharmacia) was used to express and partially purify the poxvirus phosphatases vaccinia H1L and myxoma I1L, and a myxoma I1L mutant in which the catalytic cysteine residue, Cys110, is replaced by a serine (I1L-C110S). To create I1L-C110S, the codon TGT for Cys110 was mutated to TCT, creating Ser110, using oligonucleotide-directed mutagenesis. In order to clone the three proteins in-frame into the BamHI site of pGEX-3X (Pharmacia), PCR was performed with vaccinia or myxoma DNA using SFV M1L 3' primer and a 5' primer (5'-GGCCGGATCCAAATGGATAAGAAAAG-3') which caused a single base change of T→C in order to change an in-frame TAA codon, located directly

upstream of the initiating ATG, to a CAA codon. The plasmids were designated pGSTH1L, pGSTI1L, and pGSTI1Lm.

To construct the myxoma virus I1L phosphatase disruption plasmid, two I1L internal primers were made, each containing a complementary linker region with an internal BglII restriction site. The primers were designated PIN (5'-GACCAAGATCTCGTACGATTCCCGATGTAGGAGC-3') and PIC (5'-GTACGAGATCTTGGTCGTATGGCCGGTGTAAATC-3'). Two separate PCR reactions were performed, with SFV M1L 5' and PIN primers, and SFV M1L 3' and PIC primers. The 151 bp N-terminal and 190 bp C-terminal fragments were digested with BglII, ligated together with T4 DNA ligase, and amplified using the SFV M1L 5' and 3' primers. The resulting 341 bp fragment was digested with XhoI & BamHI, and cloned into pBluescript, creating pΔI1L. pΔI1L was cleaved with BglII, and ligated to a 3.15 kbp BglII cassette containing the *E. coli* β-galactosidase gene under the control of the vaccinia p11 promoter (p11-LacZ) (1). Two disruption plasmids were constructed containing both orientations of the p11-LacZ cassette, designated pI1L-LacZa and pI1L-LacZb.

To create the M-T7 disruption plasmid, the myxoma virus BamHI K fragment (14), containing complete M-T5, M-T6 and M-T7 open reading frames plus flanking sequences, was cloned into pBluescript, creating the plasmid pBamK. A 1.9 kbp BamHI-SstII segment of pBamK containing the complete M-T7 open reading frame (792 bp) flanked by the partial open reading frames of M-T8 and M-T6, was blunt-end cloned into the SmaI site of pBluescript, creating the plasmid pKMBD-8. A 372 bp SmaI-NruI fragment within the M-T7 gene was deleted and, following the addition of BglII linkers, the deleted M-T7 coding sequence was replaced with a BglII cassette containing a dominant selectable marker, the *E. coli* guanosine phosphoribosyl transferase gene driven by the vaccinia 7.5K promoter (p7.5Ecogpt) (3). The resulting plasmid, pMT7G-4,

contained p7.5Ecogpt inserted into the SmaI-NruI deletion of the M-T7 open reading frame such that the selectable marker would be transcribed in the same direction as the M-T7 gene.

To create the M-T5 disruption plasmid, PCR amplification of pBamK DNA was used to create the fragments M-T5L and M-T5R, using the following primer pairs: M-T55' (GCCGGATCCGGACGAGGATGGATC) & Del5' (CTACGCTGCAGGACTGGCGCCATCGCTTGAC) and S-T53' (CCGGATCCTACGCATGTAACTTC) & Del3' (AGTCCTGCAGCGTAGGAATGTATAAGGGAAC), respectively. Del5' and Del3' were engineered to contain a complementary linker region with an internal PstI site. Amplified M-T5L and M-T5R fragments were mixed and subjected to one round of PCR in the absence of primers, followed by 25 rounds of PCR in the presence of M-T5 5' and S-T5 3' primers. The resulting fragment was cloned directly into the pT7Blue T-vector (Novagen), followed by subcloning of an XbaI/EcoRI fragment into pBluescript, creating the plasmid pMT5del. A PstI p7.5Ecogpt cassette was inserted into pMT5del, creating pMT5gpt.

Construction of Recombinant Myxoma Viruses.

The recombinant phosphatase disruption virus was constructed from wild type myxoma virus (strain Lausanne) using the insertion vector plasmids pI1L-LacZa and pI1L-LacZb. Selection for insertion of the lacZ cassette using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was performed as previously outlined (13) with the exception that 1μg of intact plasmid was utilized in the infection-transfection procedure. Individual blue plaques were harvested, and subjected to SDS-pronase treatment in which cells were incubated at 37°C overnight in 10mM Tris pH7.5, 10mM EDTA, 0.5% SDS and 300μg pronaseE, followed by phenol/chloroform extraction and ethanol

precipitation, in order to obtain viral DNA for PCR analysis. All samples were PCR amplified with three separate primer sets: SFV M1L 5' and 3' primers to detect wild type myxoma I1L alleles, SFV M1L 5' primer and a lacZ 3' reverse primer (5'-ACCTTATTTATCAGCCG-3') to detect myxoma I1L disruption alleles, and 5' and 3' primers directed towards the myxoma T7 open reading frame as a DNA control. The lacZ 3' reverse primer lies 530 nucleotides from the termination codon within the coding region of lacZ, and is designed to prime towards the 3' end of the lacZ gene.

M-T7$^-$ and M-T5$^-$ mutant myxoma viruses were constructed using a mycophenolic acid selection procedure (3). A recombinant M-T7/p7.5Ecogpt fragment, designated M-T7gpt, was amplified from pMT7G-4 using primers directed towards the 5' (5'-AGGTCGACATGGACGGGAGACTGGTGTTTC-3') and 3' (5'-AGGAATTCTTATTCGAAGACATCTTCCAC-3') ends of the M-T7 open reading frame. M-T7gpt was transfected (Lipofectin, GIBCO-BRL) into BGMK cells infected with vMyxlac at a multiplicity of infection of 0.1, creating vMyxlac-T7gpt. Similarly, pMT5gpt was transfected into both wild-type myxoma virus and vMyxlac infected cells, creating the recombinant viruses vMyxT5$^-$ and vMyxlacT5$^-$, respectively. After 24 hours infectious virus was isolated, and subjected to three passages under mycophenolic acid selection (25µg/ml mycophenolic acid, 20µg/ml xanthine, 15µg/ml hypoxanthine). Individual foci were then subjected to three rounds of plaque purification, and isolates were grown up for analysis and stored at -70°C for further use.

Two revertant viruses, vMyxT7rev and vMyxT5rev, in which the complete M-T7 or M-T5 open reading frames were restored back into a wild-type myxoma background, were created from vMyxlac-T7gpt and vMyxlacT5$^-$, respectively. Co-transfection of pMyS2a, which contains wild-type myxoma sequences surrounding the location of integration of the lacZ gene used to create vMyxlac

(13), and pBamK into vMyxlac-T7gpt or vMyxlacT5$^-$ infected cells yielded approximately 5% foci which remained white upon staining with X-gal. Within this population, indicative of removal of the lacZ gene, approximately 50% of virus clones now contained an intact M-T7 or M-T5 open reading frame, as confirmed by PCR analysis.

PCR analysis with either M-T7 or M-T5 primers was used to confirm both the absence of an intact M-T7 or M-T5 open reading frame in the disruption viruses, or the presence of an intact M-T7 or M-T5 open reading frame in the revertant viruses. To obtain viral DNA suitable for PCR analysis, virally infected cells from a single well of a 6-well multi-dish were harvested, resuspended in 5mM Tris pH 8, 5% NP-40, 5% Tween-20, 0.5 mg/ml proteinaseK and incubated at 55°C for a minimum of 2 hours. Following inactivation of proteinaseK by boiling, the mixture was subjected to phenol:chloroform extraction and the DNA stored at 4°C for further use.

RNA Analysis.

Subconfluent monolayers of BGMK cells in 100mm dishes were infected with vMyxlac virus at an moi of 30. RNA from infected cells was extracted at various time points post-infection using guanidine thiocyanate, as previously described (10). For Northern blotting analysis, 3µg purified viral RNA was prepared in 3-(N-Morpholino) propane-sulfonic acid (MOPS) buffer (20mM MOPS, 5mM sodium acetate, 1mM EDTA) containing 50% deionized formamide and 20% formaldehyde. Samples were incubated at 55°C for 15 minutes, cooled on ice, and loaded onto a 1% agarose gel containing 1x MOPS buffer, 2% formaldehyde, and 0.5mg/ml ethidium bromide. RNA was transferred to nitrocellulose via capillary action. For probing transcription of the I1L and M-T7 genes, random priming was performed using either the myxoma I1L or M-T7

PCR product, respectively, with [α-^{32}P] dCTP (Amersham) and random primer (Boehringer Mannheim). Results were visualized with autoradiography.

For primer extension analysis of the M-T7 gene, a 22 base oligonucleotide primer, (5'-AAGTCGTAGGACGTAAGGCGTA-3'), was end-labeled using [γ-^{32}P] ATP (ICN) and T4 kinase. The primer was constructed to complement the M-T7 coding sequence, with its 3' end residing 52 bases downstream of the initiating ATG. Primer extensions were performed as described in Sambrook et al. (15) using 5µg viral RNA, and Superscript reverse transcriptase (Gibco BRL). DNA sequencing was performed by adding one of four ddNTP mixtures (for example, ddATP: 1mM each dGTP, dTTP, dCTP, 0.5 mM dATP, 0.125mM ddATP) to annealed RNA/primer, and incubating at 55°C for 45min. All primer extension reactions were subjected to sequencing gel analysis, and visualized with autoradiography.

Expression of Vaccinia Virus and Myxoma Virus Phosphatases.

pGSTH1L, pGSTI1L, and pGSTI1Lm were transformed into *E.coli* BL21(DE3)pLysS (19) for expression studies. Cultures were grown at 30°C until OD_{600}=1.5, at which time they were induced with 0.1mM IPTG and incubated a further 3 hrs. Cells were harvested by centrifugation, resuspended in phosphate buffered saline (PBS) containing 1mg/ml lysozyme, incubated on ice for 45min, and re-centrifuged. $MgCl_2$ to 8mM was added to bacterial lysates which were treated with 10 µg/ml DNAseI on ice for 1hr. Inclusion bodies were removed by centrifugation at 10,000g for 10min at 4°C. Bacterial supernatants were incubated with Glutathione Sepharose 4B (Pharmacia) and batch purified as per manufacturers protocol. Elution was performed with 10mM reduced glutathione in 50mM Tris-HCl pH 8.0. Free glutathione was removed by dialysis against 50mM Tris-HCl (pH8.0), 150mM NaCl, and 0.1% β-mercaptoethanol.

Dialyzed samples were stored at -20°C in 50% glycerol. Protein concentrations were determined using BioRad Bradford assay.

Phosphatase Assays

Hydrolysis of p-nitrophenyl phosphate (pNPP), a chromogenic substrate for tyrosine-specific phosphatases, was performed in 100µl of solution containing 25mM imidazole pH7.2, 1mM EDTA, 0.1mM DTT, 10mM pNPP and expressed GST-fusion enzyme, at 30°C for 30 min. The reaction was stopped by the addition of 5µl 1M sodium carbonate. Absorbance at 410nm was measured using a Dynatech MR600 automated microplate spectrophotometer.

Tyrosine phosphorylation of myelin basic protein (MBP) was performed overnight at 30°C with p56*lck* immunoprecipitated from $5x10^5$ LSTRA cells and 100µCi [γ-^{32}P]ATP (ICN) in a total volume of 30µl in 10mM piperazine-N,N'-bis [2-ethane-sulfonic acid] pH7.1/10mM $MnCl_2$. Tyrosine-phosphorylated MBP was recovered after trichloroacetic acid precipitation in the presence of bovine serum albumin carrier protein. Dephosphorylation assays were performed essentially as described (18). Briefly, 10000 cpm of tyrosine-phosphorylated MBP was mixed with increasing amounts of GST-fusion enzyme in a total volume of 50µl in 25mM imidazole pH7.2/5mM EDTA/100mM DTT. After 30 min at 30°C the reactions were stopped with 0.85 mls of acidic charcoal mixture. After 10 min. at room temperature the charcoal was pelleted by centrifugation and 600µl of the supernatants quantitated for ^{32}P release, by Cerenkov counting.

Serine phosphorylation of MBP and casein was performed by incubating 100µg of protein with 300µCi [γ-^{32}P]ATP and 10 units protein kinase A (Sigma) in 50µl solution containing 20mM Tris pH 7.4, 1mM DTT, 100mM NaCl and 12mM $MgCl_2$ at 30°C overnight. Serine-phosphorylated MBP/casein was

precipitated by the addition of 0.1ml of 5mg/ml BSA and 0.5ml of 20% TCA in 20mM NaH_2PO_4, washed three times with 95% ethanol, and resuspended in 100µl 0.2M Tris pH 8.0. Assays were performed by incubating $1x10^5$ cpm phospho-MBP/casein with increasing amounts of enzyme in 50µl reactions containing 25mM imidazole pH7.2, 1mM EDTA and 0.1mM DTT for 30 min at room temperature. Reactions were stopped with acidic charcoal and released ^{32}P quantitated as outlined above.

Purification of Myxoma Virus M-T7 Protein.

Roller bottles of BGMK cells (4 roller bottles at $2x10^7$cells/roller) were incubated for 1hr with vMyxlac at an moi of 3. After extensive washing with PBS to remove unabsorbed virus and serum, fresh medium (no serum) was added. The medium containing secreted proteins were harvested 4hrs later (early supernatant). Cells were replenished with fresh medium (no serum), and secreted proteins were re-harvested 16hrs post-infection (late supernatant). Early and late supernatant samples were pooled, subjected to ammonium sulfate fractionation, and proteins which precipitated between 30% and 50% ammonium sulfate, which contained the bulk of M-T7, collected. Precipitated proteins were resuspended in 20mM Bis-Tris pH 6.0, loaded on a Q Sepharose FAST FLOW column (Pharmacia), and eluted with a 0-300mM NaCl gradient, in which M-T7 eluted in a broad peak between 150-180mM NaCl. Fractions containing the M-T7 protein were identified by SDS-PAGE, pooled, precipitated with 50% ammonium sulfate, and desalted using an Econopac 10-DG column with 20mM Hepes pH 7.9 and 1mM EDTA buffer. The resulting M-T7 protein preparations were stored at 4°C.

Western Blotting Analysis with M-T7 Antibody.

The glutathione S-transferase gene fusion system (Pharmacia) was used to express M-T7 for polyclonal antibody production. An M-T7 fragment amplified by PCR was cloned in frame into the expression vector pGEX-2T, creating the plasmid pGST7. pGST7 was transformed into *E. coli* BL21(DE3)pLysS (19), and the inclusion bodies containing the bulk of the M-T7 fusion protein isolated as described above. For the immunization of two rabbits, lyophilized acrylamide gel slices containing denatured M-T7-GST fusion protein were prepared for use as antigen, as described elsewhere (6). Polyclonal antisera from one rabbit (E266) was further subjected to affinity purification against immobilized denatured M-T7-GST fusion protein as outlined elsewhere (6) Detection of M-T7 protein in supernatants prepared from virus-infected cells was performed by Western blotting analysis, as described previously (17), with a primary antibody dilution of 1:1000.

Generation of Cellular Supernatants.

BSC-1 and BGMK cells were either mock infected or infected with ectromelia virus (BSC-1) or vMyxlac, SFV, vaccinia, cowpox, or rabbitpox viruses (BGMK) at an moi of 5-10. After 1 hr at 37°C, the monolayers were washed twice to remove unabsorbed virus, and resupplemented with serum-free media for a further 8 hours (BSC-1) or 24 hours (BGMK). Supernatants were collected, centrifuged at 5,000xg for 15 minutes to remove cellular debris, and concentrated using Amicon Centriprep-10 filters.

Chemical Crosslinking Assays.

Rabbit, human, and murine IFN-γ (Genentech) were labeled *in vitro* with protein kinase A and [γ-^{32}P] ATP, as previously described (9), with the

exception that 5µg of IFN-γ was phosphorylated at 30°C for 30 minutes. Labeling was stopped by the addition of 500µl of 1mg/ml bovine serum albumin in 10mM sodium phosphate pH7 buffer containing 10mM sodium pyrophosphate and 10mM EDTA, and separated from unincorporated [γ-^{32}P] ATP with Presto desalting columns (Pierce). Chemical crosslinking assays were performed using either 500ng purified M-T7 protein or 10µl concentrated mock infected or virus infected supernatants. Complexes formed at room temperature within 1hr, at which time the crosslinking reagent 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (Sigma), in 100mM potassium phosphate pH 7.5, was added to 20mM. The reaction was quenched 15min later by the addition of Tris-HCl pH 7.5 to 0.1M, and the resulting protein complexes analyzed by SDS-PAGE, and visualized by autoradiography. For competition binding assays, crosslinking was performed as described above, with the simultaneous addition of increasing amounts of unlabeled competitor IFN-γ.

Solid Phase Binding Assay.

Falcon immunoplates were coated overnight with 50ng M-T7 protein in 50µl PBS at 4°C. The next day, wells were blocked at room temperature for two hours with 5% skim milk powder in PBS containing 0.1% Tween 20, followed by binding of [^{32}P]-IFN-γ in blocking buffer for one hour. Following three washes with PBS, the wells were removed and counted by Cerenkov counting. To determine the specific binding of IFN-γ to M-T7, binding in the presence of 100-fold excess cold IFN-γ was subtracted from total binding. Non-specific binding routinely represented less than 5% of total binding. All assays were performed in triplicate. Binding of [^{32}P]-IFN-γ to M-T7 was analyzed by the method of Scatchard (16).

Bioassay in Tissue Culture Cells.

60mm dishes seeded to confluency with RK13 cells, BGMK cells, or L929 cells were incubated overnight in medium containing rabbit, human, or murine IFN-γ (Genentech), respectively, with increasing amounts of M-T7 protein. The next day, medium was removed, and monolayers were infected with 100 plaque forming units of VSV for 1hr, then overlaid with 1% low melting point agarose in complete medium. The number of VSV plaques were scored 24hr to 48 hr post-infection, and compared with the uninhibited control, in which cells were pre-incubated with only control medium.

Binding of Interferon-γ to the Surface of Uninfected Cells.

Binding of radiolabeled human, rabbit or murine IFN-γ to cells in monolayer culture was performed essentially as described (24). Briefly, $1x10^4$ cells seeded in 24 well dishes were washed with ice cold serum-free medium, followed by the addition of increasing amounts of [^{32}P]-IFN-γ in 100µl serum-free medium, either alone or in the presence of 100-fold excess unlabeled IFN-γ. The cultures were incubated at 4°C with gentle rocking for 1hr, at which time the monolayers were washed 3x with PBS to remove unbound [^{32}P]-IFN-γ. Cells were removed with 0.15M sodium chloride and 0.015M sodium citrate, and the radioactivity in the samples was determined by Cerenkov counting. Specific binding was calculated by subtracting nonspecific binding in the presence of excess unlabeled IFN-γ from total binding. All assays were performed in triplicate. [^{32}P]-IFN-γ binding to RK13 cells was analyzed by the method of Scatchard (16).

Quantitation of M-T7 Protein During Myxoma Virus Infection.

Supernatants of BGMK cells infected with vMyxlac virus (moi of 30) were harvested at various times post-infection and subjected to SDS-PAGE analysis, and visualized by silver staining. To quantitate the amount of M-T7 produced during myxoma virus infection, M-T7 bands were scanned using a Chromoscan3 densitometer, and compared with purified M-T7 standards. Inhibitory units (I. U.) were defined as the amount of M-T7 (ng) required to inhibit 1 unit of rabbit IFN-γ, based on 50% inhibition in a VSV inhibition bioassay.

Radiolabeling of Secreted Viral Proteins.

BGMK cells ($6x10^5$ per sample) were infected with vMyxlac virus at an moi of 30. At 1.5hr (for labeling of early viral proteins) or 8hr (for labeling of late viral proteins) post-infection, cells were washed with PBS, pulsed for 30 min with 200μCi [^{35}S]cysteine/[^{35}S]methionine (Translabel, ICN) in 400ml cysteine/methionine deficient medium (Gibco), washed 3x to remove unincorporated label, then placed in complete medium. To detect labeled viral proteins secreted within the indicated one hour time frame, complete medium was replaced with serum-free medium for one hour prior to harvesting of labeled supernatants, with the exception of the first time point of 2.5hr post-infection, in which case serum-free medium was added for 30 min after labeling. To detect accumulated labeled secreted viral proteins, cells were infected and labeled as above, and serum-free medium was added to washed cells following labeling. Cellular supernatants containing labeled, secreted viral proteins were harvested at various time points post-infection, centrifuged at 5000xg to remove cellular debris, subjected to SDS-PAGE analysis, and visualized by autoradiography.

Metabolic Labeling of Infected Cells.

$1x10^6$ cells were infected with virus at an moi of 10 for one hour, followed by addition of medium containing serum. At indicated times after viral absorption, the medium was removed and cells washed once with cysteine/methionine-free medium (ICN). Cells were then incubated with 50 µCi of [^{35}S]cysteine/[^{35}S]methionine (ICN) in cysteine/methionine-free medium for 30 min. Cells were lysed directly into 50 µl of SDS-PAGE buffer, and samples analyzed by SDS-PAGE followed by autoradiography.

Apoptosis Assays.

Apoptosis was monitored in cultured RL5 and primary rabbit peripheral mononuclear leukocytes by fluorescence activated cell sorting analysis based on the TUNEL method of detecting dNTP-binding to 3'-hydroxyl ends of fragmented DNA (5). Peripheral blood cells were isolated by layering rabbit blood over an equivalent volume of Ficoll:Paque and centrifuging at 1500 rpm for one hour. The mononuclear cell fraction was collected, washed twice with PBS, and resuspended in RPMI supplemented with 10% FCS. Following a four hour incubation, adherent and non-adherent cell populations were obtained. For the TUNEL reaction, $1x10^6$ cells were mock infected or infected with virus at a multiplicity of infection of 10 for one hour, and harvested 12-16 hours post-infection. Infected cells were washed once in PBS containing 1% FCS (PBS/FCS), fixed in 200 µl 2% paraformaldehyde in PBS for 30 min at room temp with gentle agitation, rewashed in PBS/FCS, then permeabilized with 100 µl cold 0.1% Triton X-100, 0.1% sodium citrate for 2 min on ice. Following two washes with PBS/FCS, cells were incubated for one hour at 37°C with 0.6 nmol Fluorescein-12-dUTP (Boehringer Mannheim), 3 nmol dATP, 1mM $CoCl_2$, 25 units terminal deoxynucleotidyl transferase (TdT, Boehringer Mannheim), and

TdT buffer (30 mM Tris pH7.2, 140 mM sodium cacodylate) in a total volume of 30 µl. The reaction was stopped by adding 3 µl 200 mM EDTA, cells were washed once in PBS/FCS, and resuspended in 0.5 ml PBS. Flow cytometric analysis was performed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) equipped with an argon-ion laser with 15 mW of excitation at 488 nm. Data was acquired on 10,000 cells/sample with light scatter signals at linear and fluorescence signals at logarithmic gain.

DNA fragmentation assays were performed on $2x10^6$ RL5 cells infected with virus at a multiplicity of infection of 10 for one hour. At the indicated times post-infection, cells were lysed in 1% SDS, 100 mM NaCl, 1 mM EDTA and 10 mM Tris pH7.5. Lysed cells were treated with 0.5 mg/ml proteinase K for one hour at 55°C followed by treatment with 0.5 mg/ml RNase for one hour at 37°C. DNA was precipitated with 2.5 vol 95% ethanol, resuspended in 10 mM Tris pH8.0, and subjected to agarose gel electrophoresis in the presence of 0.5 µg/ml ethidium bromide.

Single Step Growth Analysis in Tissue Culture.

Cultures of RK13 and RL5 cells were infected with either vMyxlac and vMyxlac-T7gpt (RK13) or vMyxlac and vMyxlacT5$^-$ (RK13 and RL5) at a multiplicity of infection of 5 for one hour. The inoculum was removed, the cells washed with serum-free medium three times, replaced with medium supplemented with serum and cultures were harvested at various times post inoculation. Virus titres were determined by a plaque assay on RK13 cells followed by X-gal staining of fixed monolayers, as outlined (13). All growth analyses were performed in triplicate.

Infection of Rabbits With Recombinant Myxoma Viruses.

Female New Zealand White rabbits (*Oryctolagus cuniculus*) were obtained from a local supplier and housed in C-level containment facilities under the guidelines of Canadian Council on Animal Care. Injections were performed intradermally in each thigh with 1000 pfu virus per site. Rabbits were monitored daily for symptoms of myxomatosis, including appearance of primary and secondary tumors, conjunctivitis and rhinitis, and responsiveness to stimuli (4, 12).. Rabbits which became moribund were sacrificed with euthanyl administered intravenously after anesthesia. For gross pathological analysis, in one study 13 rabbits were inoculated with vMyxlac-T7gpt, 3 with the vMyxlac parental virus and 2 with the T7 revertant virus, while in a second study 8 rabbits were inoculated with vMyxT5$^-$, 4 with the wild-type vMyx parental virus and 2 with the T5 revertant virus. In previously published studies (11, 13, 21), in which a minimum of 18 rabbits have been systematically tested with vMyxlac, we have observed a mortality rate of approximately 95% with this virus. Since this overall lethality is slightly less than the 100% ascribed to the original Lausanne isolate of myxoma, we have now classified vMyxlac as possessing grade II virulence, using the original definition of Fenner (4).

Histological Analysis and Immunohistological Staining.

For histological studies, six rabbits each were inoculated with vMyxlac, vMyxlac-T7gpt, or vMyxlacT5$^-$ as described above. Two rabbits from each group were sacrificed at days 4, 7 and 10 post-infection, a complete post-mortem examination was performed, and tissue sections removed and placed in neutral buffered formalin. Samples were embedded in paraffin, cut into 5 μm sections and stained with hematoxylin and eosin for viewing by light microscopy. For immunohistological studies with vMyxlac and vMyxlac-T7gpt,

the ExtrAvidin Peroxidase Staining Kit (Sigma Immunochemicals) was utilized, as per manufacturers specifications. Briefly, paraffin was removed with xylene, and samples rehydrated before being counterstained with Harris Hematoxylin. Samples were then subjected to treatment with 3% hydrogen peroxide, followed by staining with mouse monoclonal CD43 or anti-β–galactosidase antibodies (Spring Valley and Promega, respectively), at a dilution of 1:500. The CD43 antibody recognizes rabbit T cells, monocytes and macrophages, while the β–galactosidase antibody detects the protein product of the *E. coli* β–galactosidase cassette expressed from the vMyxlac and vMyxlac-T7gpt viruses. Following application of affinity purified biotinylated goat anti-mouse immunoglobulin and ExtrAvidin-Peroxidase, samples were subjected to the substrate DAB (Sigma). Finally, samples were dehydrated, mounted in Permount, and photographed using Kodak Ektachrome 64T Colour Reversal Film.

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CHAPTER III

The Myxoma Virus Soluble Interferon-γ Receptor Homolog, M-T7, Inhibits Interferon-γ in a Species Specific Manner[1]

[1]All data contained herein have been published: Mossman, K., Upton, C., and McFadden, G. (1995) *Journal of Biological Chemistry* **270**: 3031-3038.

Introduction

IFN-γ is a potent immunomodulatory cytokine primarily produced by activated T lymphocytes and natural killer cells (45). While IFN-γ was discovered by virtue of its anti-viral activities, it also serves critical functions as an immunoregulator in the presence and absence of pathogenic challenge (8, 10). IFN-γ exerts pleiotropic effects on the immune system through ligand-dependent activation of the IFN-γR (10). The human and murine IFN-γRs have been extensively characterized, and are known to bind IFN-γ with high affinity in a species specific manner (1, 17). The importance of IFN-γ within the immune system is exemplified by the observation that IFN-γ is the primary cytokine mediator of innate resistance to both viral and non-viral pathogens (5, 19). IFN-γ functions to combat viral infections by inducing anti-viral pathways and by modulating cellular immune responses to viral challenge.

To overcome the anti-viral and immunoregulatory effects of IFN-γ, many viruses have evolved both extracellular and intracellular anti-interferon strategies (16, 22, 24, 29, 38). Poxviruses, a family of large, double stranded DNA viruses which replicate within the cytoplasm of host cells (31), were the first viruses found capable of interrupting the extracellular ligand-dependent triggering of the IFN-γRs, thus preventing signal transduction from an extracellular location (44). In particular, the *Leporipoxvirus* myxoma virus was found to express a soluble IFN-γR homolog, denoted M-T7, which has the ability to bind and inhibit the anti-viral activities of rabbit IFN-γ (44). Here, we investigate the secretion and inhibitory properties of M-T7, the myxoma soluble IFN-γR homolog, in an attempt to further understand the role this viral protein plays in combating IFN-γ during the establishment of a virus infection.

Results

Comparison of Poxviral IFN-γ Receptor Homologs

The *Leporipoxvirus* myxoma virus was recently shown to encode and express a 37 kDa soluble protein, designated M-T7, containing significant homology to the ligand binding domain of the human and murine IFN-γ receptors (44). SFV, a *Leporipoxvirus* closely related to myxoma virus, also encodes a similar IFN-γ receptor homolog, designated S-T7 (44). As the genomic sequence information from a variety of other poxviruses accumulates (15, 25-27, 37, 39), additional putative viral IFN-γ receptor homologs have been discovered (32). Included are the *Orthopoxvirus* vaccinia virus (B8R) and variola virus (B8R in strain Bangladesh-1975 and B9R in India-1967) (15, 25, 37) proteins, and the *Suipoxvirus* swinepox virus C6L protein (26). Figure III-1 illustrates the amino acid alignment of the five known poxviral IFN-γ receptor homologs with the two known mammalian IFN-γ receptors. Although the overall identity scores between the poxviral proteins and the ligand binding domain of the mammalian IFN-γRs is low (20-25%), it is important to note the conservation of cysteine residues, which for the mammalian IFN-γRs have been shown to be essential for ligand binding by stabilizing disulfide bonds (12, 14). As highlighted in Figure III-1, the myxoma M-T7, SFV S-T7 and swinepox C6L proteins contain all 8 conserved cysteine residues, while the vaccinia and variola B8R proteins have the first two cysteine residues replaced with tyrosine residues, while a single cysteine residue is situated at a unique site four amino acids N-terminal to cysteine #3 (Figure III-1). Note that all of the poxviral proteins lack transmembrane domains and are predicted to be secreted proteins. Thus, the poxviral IFN-γ binding proteins are uniformly shorter than their mammalian receptor homologs, and possess neither hydrophobic

membrane-spanning domains, nor cytoplasmic signalling domains required to initiate an IFN-γ signal transduction event.

M-T7 is Expressed From an Early Poxviral Promoter

The myxoma 37 kDa M-T7 protein was previously observed to be the major viral protein detected in medium harvested from infected cells at both early and late times of infection (44). Since some poxviral proteins, such as the 7.5kDa polypeptide of vaccinia, are expressed constitutively during infection due to the presence of overlapping early and late promoters (4), it was of interest to examine the kinetics of M-T7 expression. Northern blotting analysis, using a radiolabeled M-T7 probe, demonstrates that a single M-T7 mRNA transcript of approximately 1.1 kb from myxoma virus infected cells is present at early times (Figure III-2, lanes 2 and 3), and is still detectable at late times (lanes 4 and 5). Primer extension analysis confirms the results of the Northern blot analysis: i.e. a defined extended product can be clearly detected with RNA collected at early times post-infection (Figure III-3a, lanes 3 and 4), but is still observed at late times post-infection (lanes 5 and 6). By combining the primer extension reaction with dideoxy sequencing, we mapped the 5'-initiation sites of the M-T7 transcript to a sequence motif with all the elements of a strong poxviral early promoter (Figure III-3b and c). In contrast, the highly conserved TAAAT motif characteristically observed in most poxviral late promoters (7) is absent. Vaccinia virus early promoters (Figure III-3c) typically include a 16 bp critical region, followed by a T-rich 11 bp spacer and a 7 bp initiation region, where transcript start sites are clustered at purine residues (6). The M-T7 critical region is highly homologous to the critical region experimentally engineered in vaccinia virus (6) to be substantially stronger than natural early promoters (Figure III-3c). Transcription of M-T7 predominantly initiates at a single purine

(adenine) 10 nucleotides upstream of the ATG, although three surrounding bases are also used in initiation, to a lesser extent (refer to Figure III-3 b and c). Furthermore, no evidence of a 5'-poly(A) leader, characteristic of poxviral late mRNAs (7), was detected when primer extensions were performed with mRNA collected at early or late times (data not shown).

M-T7 is Efficiently Secreted as a Stable Soluble Protein

Since M-T7 is expressed from a typical early poxviral gene, and yet is the major secreted protein observed in supernatants harvested at both early and late times of a myxoma virus infection (44), we next studied the kinetics of protein secretion from myxoma virus infected cells. Both the kinetics of M-T7 secretion and the stability of the secreted M-T7 protein were assayed by infecting cells with myxoma virus, pulse labeling with [^{35}S]cys/met, and then following the fate of the labeled M-T7 protein. To determine the kinetics of M-T7 protein secretion during myxoma virus infection, we labeled proteins 1.5-2 hr post-infection, when M-T7 mRNA transcripts are abundant (refer to Figure III-2), then followed the secretion of proteins translated within this labeling period. Figure III-4a demonstrates that the transit time for M-T7 is greater than 30 min (lane 2), but less than 4 hrs (lane 4). Within 4 hr of translation, almost all of the M-T7 has been secreted (compare lanes 3 and 4). Cellular supernatants from myxoma virus infected cells which had been labeled with [^{35}S]cys/met at 8 hr post-infection, washed and then harvested two hours later, confirmed that M-T7 is secreted at early times, and although M-T7 mRNA is still readily detectable at these times (Figure III-2), secreted protein is not (Figure III-4a, lane 7). Analysis of the levels of total accumulated proteins following myxoma virus infection (Figure III-4b) demonstrates no significant reduction in the levels of intact M-T7 protein, indicating that the soluble mature form of M-T7 is stable. Thus, we

conclude that M-T7 is expressed as an early gene product which is secreted within 4hr of translation, but persists as a stable soluble protein well into late times of infection. Indeed, we have been unable to detect any significant degradation of mature M-T7 protein even after several days at 37°C in unfractionated supernatants (data not shown).

Quantitation of M-T7 Protein From Infected Cells

In order for M-T7 to play a significant role in the inhibition of host derived IFN-γ, sufficient amounts of the soluble IFN-γR homolog to outcompete cellular receptors must be secreted by myxoma virus during infection. Quantitation of M-T7 secreted at various times during myxoma virus infection demonstrated that, indeed, the amount of M-T7 secreted vastly exceeds the number of cellular IFN-γRs at the cell surface (Table III-1). In accordance with the kinetic studies outlined above, the majority of M-T7 is secreted by 4hr post-infection, with a slight increase in accumulation seen until 8hrs post-infection, at which time the total amount of M-T7 slowly decreases. While the amount of IFN-γ produced locally during viral infection has yet to be determined, the amount of M-T7 produced per cell is significant when compared to the level of IFN-γRs expressed on cell membranes. IFN-γ receptive cells are known to express 200-25,000 IFN-γRs per cell (10), which even at the maximal level of expression is three orders of magnitude less than the amount of M-T7 secreted by 4hr post-infection. Furthermore, quantitation of the inhibitory potential of M-T7 demonstrated that 10^6 cells secrete sufficient M-T7 to neutralize 2000-3000 units of rabbit IFN-γ (Table III-1). Thus, biologically significant amounts of M-T7 are produced during a myxoma virus infection.

M-T7 is a Specific Inhibitor of Rabbit IFN-γ

Previously, we showed that M-T7 was extremely efficient in abrogating the antiviral state induced by rabbit IFN-γ on RK13 cells (44). To determine the specificity of M-T7 binding, we pretreated rabbit, murine, and primate cell lines with sufficient cognate IFN-γ to induce the antiviral state, either in the presence or absence of M-T7 protein. Following pretreatment, cells were infected with VSV, and the number of virus plaques counted. The number of plaques were then compared with those counted on control monolayers in which no IFN-γ was added. As shown in Table III-2, as the molar ratio of M-T7 incubated with rabbit IFN-γ on RK-13 cells is increased, the extent of induction of the antiviral state becomes progressively inhibited, permitting the same level of VSV plaque formation, with 405 pmol M-T7, as seen with monolayers not treated with rabbit IFN-γ. However, with primate and murine cells pretreated with human and murine IFN-γ, respectively, increasing amounts of M-T7 did not affect the induction of the antiviral state, and the number of VSV plaques did not increase from the number detected with treatment of IFN-γ alone. Thus, M-T7 is species specific and can prevent the induction of the antiviral state induced by rabbit IFN-γ, but not human or murine IFN-γ.

To further confirm that the species specific ability of myxoma M-T7 protein to inhibit the biological activity of IFN-γ, a series of competition binding assays, as assessed by the chemical cross-linking of M-T7 to radiolabeled IFN-γ, were performed. The 37 kDa M-T7 protein readily binds to [^{32}P]rabbit IFN-γ, forming a complex which migrates with an apparent molecular mass of 48 to 50 kDa. As shown in Figure III-5a (lane 6), M-T7/IFN-γ complexes and small amounts of higher molecular weight complexes are observed in these cross-linking assays, along with IFN-γ monomers, dimers, and trimers, which are commonly observed in cross-linking assays involving radiolabeled IFN-γ (43). However, with the

addition of increasing amounts of unlabeled competitor rabbit IFN-γ, only the intensity of the M-T7/IFN-γ complex decreases, and at higher cold competitor ratios IFN-γ trimers, which co-migrate with M-T7/IFN-γ heterodimers, become more prominent. If unlabeled human or murine IFN-γ are substituted as the cold competitor (Figure III-5b and c, respectively), the amount of the labeled M-T7/IFN-γ complex does not decrease, even at 500x molar excess. We have also been unable to detect significant amounts of cross-linked M-T7/IFN-γ complex when murine or human IFN-γ is used as the radiolabeled ligand (data not shown). Thus, human and murine IFN-γ cannot compete with rabbit IFN-γ for binding to M-T7, further confirming the hypothesis that M-T7 has evolved to specifically inhibit rabbit IFN-γ.

Scatchard Analysis of M-T7 Binding to Rabbit IFN-γ

In order to quantitate the affinity of M-T7 for rabbit IFN-γ, solid phase binding assays were performed, followed by Scatchard analysis. Saturable binding was observed between M-T7 and [^{32}P]rabbit IFN-γ, while no binding could be observed with either radiolabeled human or murine IFN-γ (Figure III-6a). Scatchard analysis of the binding data yielded a linear plot, consistent with a single affinity binding site, with a K_d of 1.2×10^{-9} M and correlation coefficient of -0.93 (Figure III-6b). Since the K_d of binding of rabbit IFN-γ with the cognate rabbit cellular receptor had not previously been reported, binding of IFN-γ to intact rabbit RK-13 cells was also measured. Saturable binding was observed with rabbit IFN-γ, but not with human or murine IFN-γ (Figure III-7a). Scatchard analysis of the binding curve for rabbit IFN-γ gave a linear plot, with a K_d of 5.9×10^{-10} M and correlation coefficient of -0.97 (Figure III-7b).

Discussion

IFN-γ is a critical regulator of the immune system, particularly during the response to pathogenic challenge (10). The importance of IFN-γ in the response to poxvirus infections has been amply demonstrated (3, 20, 21, 23, 30, 34, 35), and thus mechanisms to combat the anti-viral effects of IFN-γ would clearly be advantageous for virus survival in vertebrate hosts. Myxoma virus, a *Leporipoxvirus*, was the first virus shown to have evolved a strategy to overcome the affects of the IFN-γ ligand prior to receptor engagement (44), by elaborating a *bona fide* soluble homolog of the mammalian IFN-γRs, named M-T7 (44). Soluble IFNγR homologs have also been described in a variety of other poxviruses, including SFV, variola virus, tanapox virus, swinepox virus, cowpox virus, camelpox virus, ectromelia virus, and all tested strains of vaccinia virus (ref. 2, 9, 15, 26, 27, 37, 44, & Chapter V). While the poxviral IFNγR homologs all contain significant homology to the mammalian IFNγR ligand binding domain, the overall extent of homology with the entire sequence of the two known mammalian receptors is relatively low. Interestingly, the percent identity between IFNγR homologs of different poxviral genera (ortho-, lepori- and sui-poxviruses) is equally low. However, conservation of the eight cysteine residues believed critical for forming stabilizing disulfide bonds (12, 13) is observed, although the vaccinia, variola and ectromelia virus homologs are noticeably missing the first two of the eight conserved cysteine residues (Figure III-1 and Chapter V). X-ray crystallography studies of the human soluble IFN-γR revealed a two domain structure, with the first two cysteine residues forming the lone disulfide bond within domain 1 (46). Mutational analysis demonstrated that disruption of the domain 1 disulfide bond completely abolishes biological activity of the receptor, while disruption of either of the remaining three disulfide bonds results in residual loss of activity (41). It will be useful to ascertain if

these two orthopoxvirus homologs have altered ligand binding properties compared with other members of the receptor family.

The mammalian IFN-γRs, through co-operation of the ligand binding and signal transducing accessory components, have been shown to possess strict species specificity for both ligand binding and ligand-dependent signalling. Since myxoma virus is known to have evolved in the tapeti, or South American brush rabbit, it was of interest to deduce whether the myxoma M-T7 soluble receptor homolog might possess the same strict species specificity towards ligand binding. Here we show that M-T7 is highly specific in binding to and abrogating the anti-viral effects of rabbit IFN-γ, and cannot bind or inhibit human or murine IFN-γ. Similar results have been found with the myxoma virus tumor necrosis factor receptor homolog (36), which inhibits rabbit tumor necrosis factor α in a species specific manner, similar to that of M-T7. This observation suggests that different poxvirus IFN-γ receptor homologs will have ligand specificities that reflect the vertebrate host(s) in which each virus has uniquely evolved. Furthermore, the ligand binding properties of soluble poxviral IFN-γR homologs would be predicted to be similar to their mammalian counterparts. We show here that M-T7 binds its ligand with a K_d comparable to the soluble mammalian receptors. Moreover, upon characterization of the rabbit RK13 high affinity IFN-γ cellular receptor, which has a K_d value similar to the human and murine cellular IFN-γRs, we demonstrate that M-T7 binding to its ligand, as with mammalian soluble receptor binding, is an order of magnitude lower than that observed with the intact cellular receptor complexes (12, 13).

Analysis of the interaction of IFN-γ with secreted versions of the mammalian IFN-γRs, described above, has demonstrated that the extracellular ligand binding domain is sufficient for IFN-γ binding (12). Naturally occurring and engineered forms of soluble IFN-γRs possess immunomodulatory

properties consistent with IFN-γ inhibition, presumably by binding and effectively sequestering the ligand away from its membrane bound receptor (12, 13, 33). Viral IFN-γR homologs presumably abrogate the effects of IFN-γ in the same fashion. In order for myxoma virus to successfully inhibit IFN-γ, sufficient amounts of M-T7 must be secreted during viral infection. Indeed, we found that biologically significant amounts of M-T7 were expressed, with respect to both the amount of M-T7 produced and its inhibitory capacity, confirming the original observation that M-T7 is a *bona fide* IFN-γR homolog.

By virtue of the amount of M-T7 expressed during myxoma virus infection, it is feasible to suggest that this abundance is a reflection of the relative importance of IFN-γ as an anti-viral cytokine. M-T7 is expressed as an early poxvirus gene product, and is efficiently secreted and extremely stable as an extracellular protein. We suspect that selection pressures on myxoma virus for replication in rabbits has resulted in efficient shuttling of high amounts of M-T7 through cellular secretory pathways, but further experiments are required in order to determine whether this represents an accelerated secretion profile or simply a strict absence of any Golgi/endoplasmic reticulum retention signals in the M-T7 protein itself.

Aside from IFN-γR homologs, poxviruses encode proteins with homologies to a number of different cytokine receptors (15, 25, 28, 38, 40). The origin of these viral cytokine receptor genes remains unproven, but it is likely that either an ancestral poxvirus acquired a progenitor cytokine binding function which subsequently evolved to mimic the ligand binding specificity of the host, or that individual poxviruses have acquired cytokine binding genes independently from their respective hosts. Myxoma virus is one of the few poxviruses for which the evolutionary host, the South American brush rabbit *(Sylvilagus brasiliensis)*, has been clearly established (11). However, the exact

evolutionary relationship between M-T7 and the rabbit IFN-γR must await the cloning and sequencing of the rabbit receptor(s). In conclusion, this work strongly suggests that careful analysis of the interaction between the poxviral IFN-γRs and IFN-γ ligands from a variety of vertebrate hosts will shed light on the evolutionary origins of poxviruses whose natural histories are still obscure.

Table III-1. Secretion of myxoma M-T7 protein.

Time (hr post-infection)	Total M-T7 Secreted		
	(I. U. / 10^6 cells)	(μg / 10^6 cells)	(molecules / cell)
2.5	450	0.48	7.81×10^6
4	2150	2.30	3.74×10^7
6	2660	2.85	4.64×10^7
8	2990	3.20	5.21×10^7
10	2710	2.90	4.72×10^7
24	2150	2.30	3.74×10^7

Tabulation of myxoma virus M-T7 levels from infected BGMK cells observed at various times post-infection. Inhibitory units (I.U.) are expressed as the amount of M-T7 (ng) required to inhibit 1 unit rabbit IFN-γ.

Table III-2. Myxoma M-T7 protein is a specific inhibitor of rabbit IFN-γ

	% Virus Yield (# of expts)		
	rabbit RK13	murine L929	primate BGMK
- IFN-γ	100	100	100
+ IFN-γ	(1.1 pmol/10^6cells)	(0.13 pmol/10^6 cells)	(1.2 pmol/10^6 cells)
M-T7 (pmol)			
405	100 +/- 5 (3)	12* +/- 9 (3)	10* +/- 7 (3)
203	nd	14 (1)	0* (1)
135	nd	nd	16 (1)
41	52 +/- 6 (3)	nd	nd
4	46 (1)	nd	nd
$4x10^{-1}$	14 (1)	nd	nd
$4x10^{-2}$	0 (1)	nd	nd
none	4 +/- 3 (3)	13 +/- 4 (3)	13 +/- 6 (3)

Percentage of virus yield based on 100% VSV yield in absence of IFN-γ or M-T7 treatment. Amount of rabbit, murine, and human IFN-γ applied to rabbit RK13, murine L929, and primate BGMK cells, respectively, is indicated. (*) indicates the presence of viral foci as opposed to fully developed viral plaques, which are not counted as plaques in this assay. nd, not determined.

Figure III-1. Alignment of soluble poxviral IFN-γ receptor homologs with the cellular mammalian IFN-γ receptors. Amino acid sequence alignment of myxoma virus M-T7 (44) with the peptide sequences of four putative poxviral IFN-γR homologs, Shope fibroma virus S-T7 (42), swinepox virus SPV C6L (26), vaccinia virus B8R (VV-B8R) (18), and variola virus Bangladesh-1975 B8R (VAR-B8R) (25), and the cellular murine (mulFNR) (17) and human (hulFNR) (1) IFN-γRs. Boxes indicate amino acid identity amongst all proteins, while (*) denotes amino acids which are conserved in at least four of the seven proteins. The disulfide-forming cysteine residues conserved between the mammalian and viral proteins are both boxed and numbered 1 to 8. The arrow indicates the location of the NH_2-terminal residue, determined by sequencing, of the mature secreted myxoma M-T7 protein, while the predicted transmembrane domains of the mammalian IFN-γRs are underlined. The full length of the human and murine IFN-γRs are 489 and 477 amino acids, respectively.

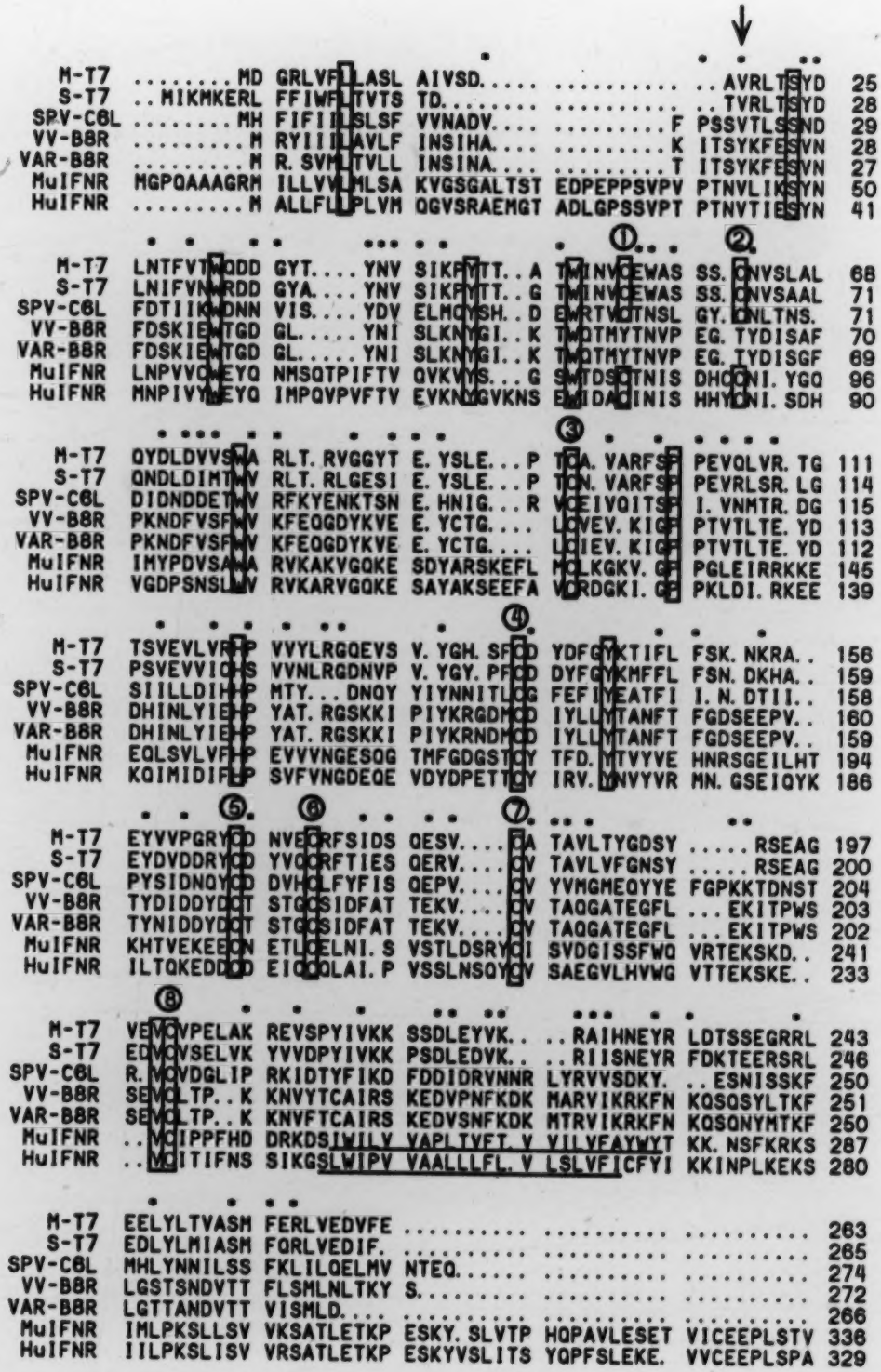


Figure III-2. Northern blot analysis of M-T7 transcripts from myxoma virus infected cells. RNA from mock infected BGMK cells (lane 1) and myxoma virus (multiplicity of infection = 30) infected cells harvested at the indicated times post-infection (lanes 2-5) was subjected to Northern blot analysis using a radiolabeled M-T7 probe. RNA marker sizes are indicated to the left of the figure.

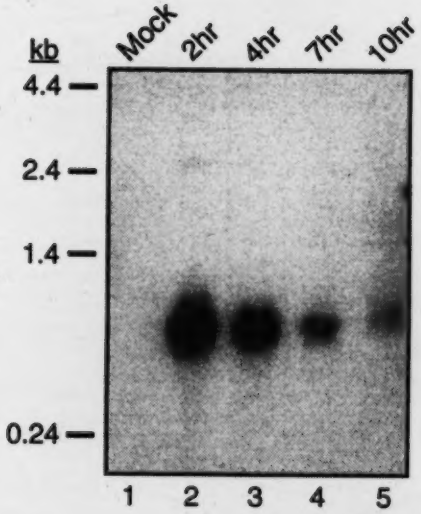
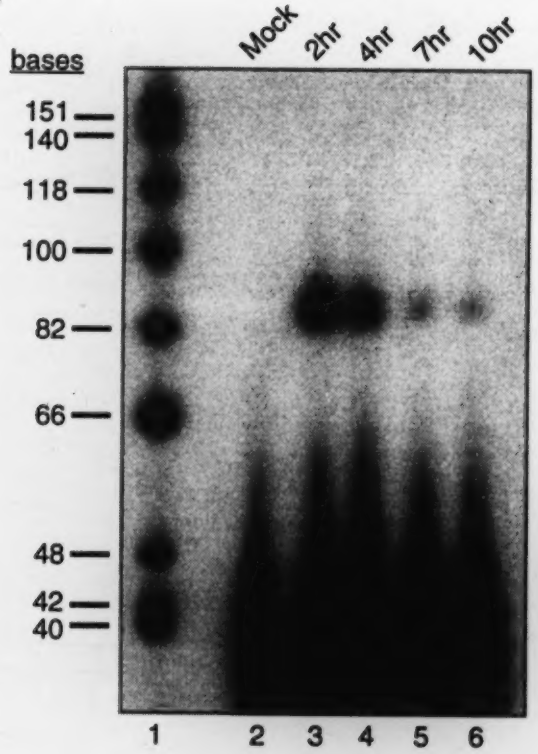
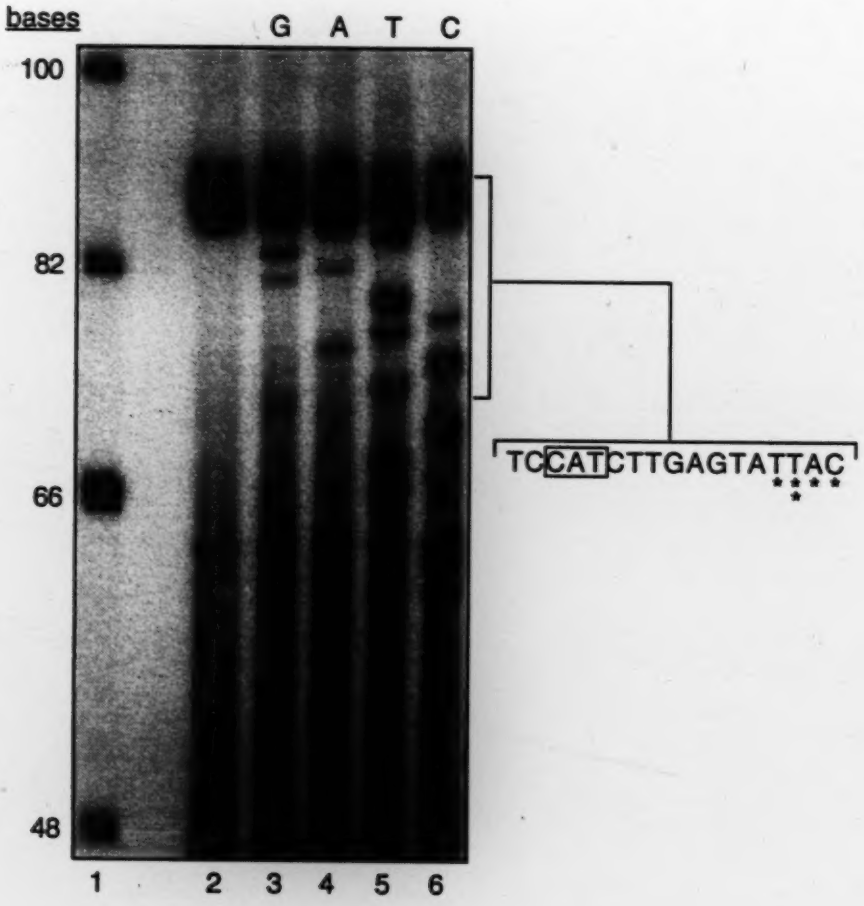

kb
Mock
2hr
4hr
7hr
10hr
4.4
2.4
1.4
0.24
1
2
3
4
5

Figure III-3. Primer extension analysis of the 5' end of the M-T7 gene. (A) hybridization of a [^{32}P] end-labelled oligonucleotide to RNA from mock infected cells (lane 2) and myxoma virus infected cells harvested at the indicated times post-infection (lanes 2-6). Size markers at the left are HinfII cut øX174. (B) dideoxy sequencing reaction using the same oligonucleotide primer as in (A), using RNA harvested 4 hours post-infection. Lane 2 is a control extension reaction in which no dideoxynucleotide is used, while the dideoxynucleotide used in each remaining reaction is indicated above the figure (lanes 3-6). Sequence of the extended product (non-coding strand) is illustrated on the right, with (*) indicating the intensity of the run-off positions. The boxed nucleotides correspond to the ATG of the coding strand. (C) promoter region upstream of the M-T7 start site (see text for details). The vaccinia consensus motifs are from Davison and Moss (6).

A.


bases
Mock
2hr
4hr
7hr
10hr
151
140
118
100
82
66
48
42
40
1
2
3
4
5
6

B.



C. M-T7 promoter

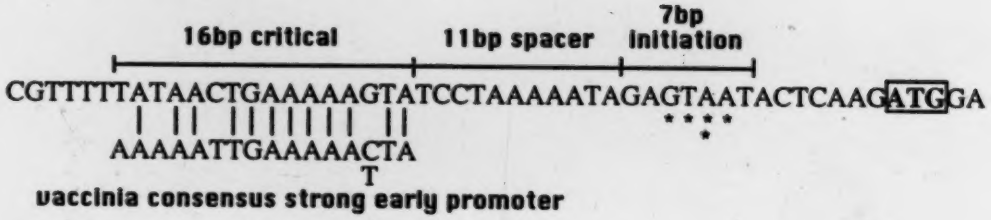
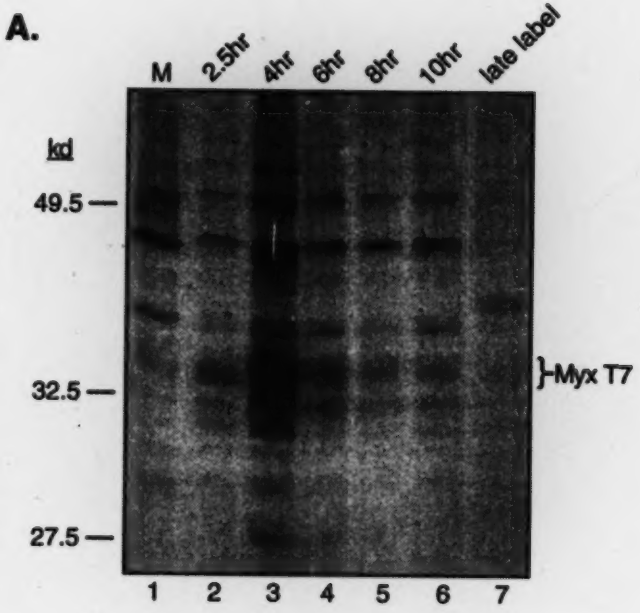


Figure III-4. Kinetics of viral protein secretion from myxoma virus infected cells. (A) [^{35}S] labeled proteins secreted from mock infected cells (lane 1) and myxoma virus infected cells (lanes 2-7) were measured. At various times post-infection, proteins secreted within a 1hr time frame ending at the indicated times were harvested. All samples were labeled with [^{35}S]cys/met for 30min at 1.5hr post-infection, with the exception of lane 7, where labeling was initiated 8hrs post-infection. (B) accumulation of [^{35}S] labeled proteins secreted from mock infected cells (lane 1) and myxoma virus infected cells at the indicated times post-infection (lanes 2-5). The location of the myxoma M-T7 protein is indicated to the right, while the location of size markers are indicated to the left.


A.
M
2.5hr
4hr
6hr
8hr
10hr
late label
kd
49.5
32.5
27.5
}Myx T7
1
2
3
4
5
6
7

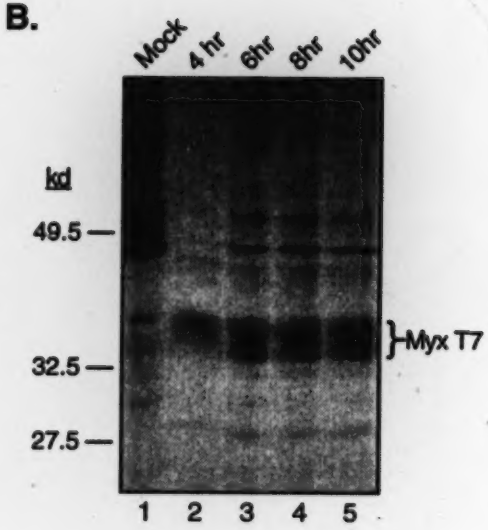

B.
Mock
4 hr
6hr
8hr
10hr
kd
49.5
32.5
27.5
}Myx T7
1
2
3
4
5

Figure III-5. Competition studies involving chemical cross-linking of [^{32}P] rabbit IFN-γ to the myxoma M-T7 protein. Competition assays of [^{32}P]rabbit IFN-γ to M-T7 were performed in the presence of increasing molar excess of unlabeled rabbit IFN-γ (panel A), human IFN-γ (panel B), and murine IFN-γ (panel C). The fold excess of unlabeled IFN-γ used as competitor is indicated at the top of the figure. The locations of monomer (M) and dimer (D) forms of the labeled IFN-γ, as well as the heterodimeric complex formed between rabbit IFN-γ and M-T7, are indicated on the right. Higher molecular weight bands of complexes containing IFN-γ are also visible. IFN-γ trimers co-migrate with M-T7/IFN-γ heterodimers (43). The locations of SDS-PAGE markers are shown on the left.

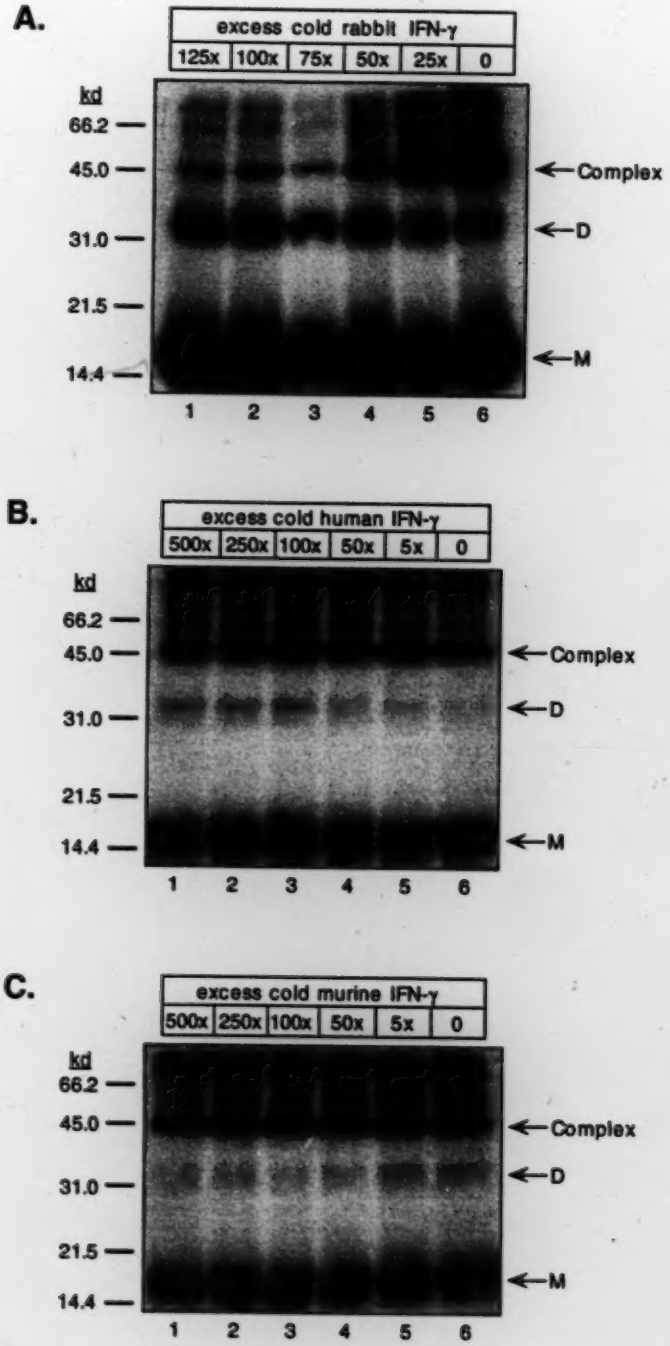
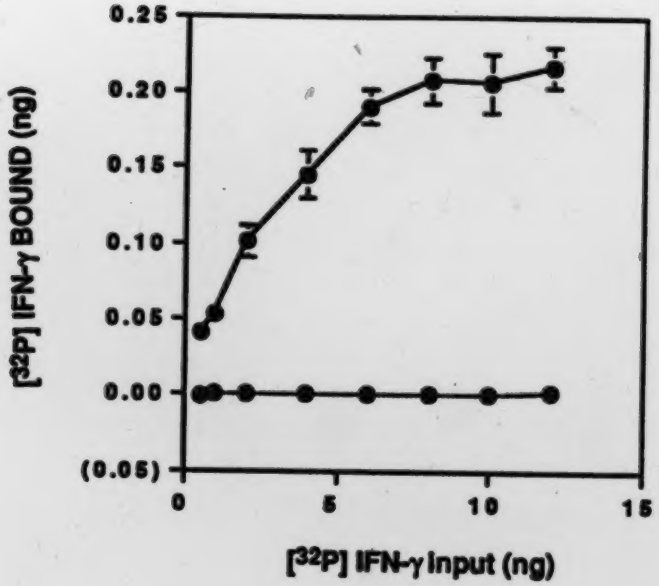

A.
excess cold rabbit IFN-γ
125x 100x 75x 50x 25x 0
kd
66.2
45.0
31.0
21.5
14.4
Complex
D
M
1 2 3 4 5 6
B.
excess cold human IFN-γ
500x 250x 100x 50x 5x 0
kd
66.2
45.0
31.0
21.5
14.4
Complex
D
M
1 2 3 4 5 6
C.
excess cold murine IFN-γ
500x 250x 100x 50x 5x 0
kd
66.2
45.0
31.0
21.5
14.4
Complex
D
M
1 2 3 4 5 6

Figure III-6. Solid phase equilibrium binding analysis of [^{32}P] IFN-γ to M-T7. (A) solid phase binding analysis of the myxoma M-T7 protein with [^{32}P] rabbit IFN-γ (●), [^{32}P] human IFN-γ (○), and [^{32}P] murine IFN-γ (+). (B) Scatchard analysis of the binding curve of [^{32}P] rabbit IFN-γ and M-T7.

A.


0.25
0.20
0.15
0.10
0.05
0.00
(0.05)
0
5
10
15
[32P] IFN-γ BOUND (ng)
[32P] IFN-γ input (ng)

B.

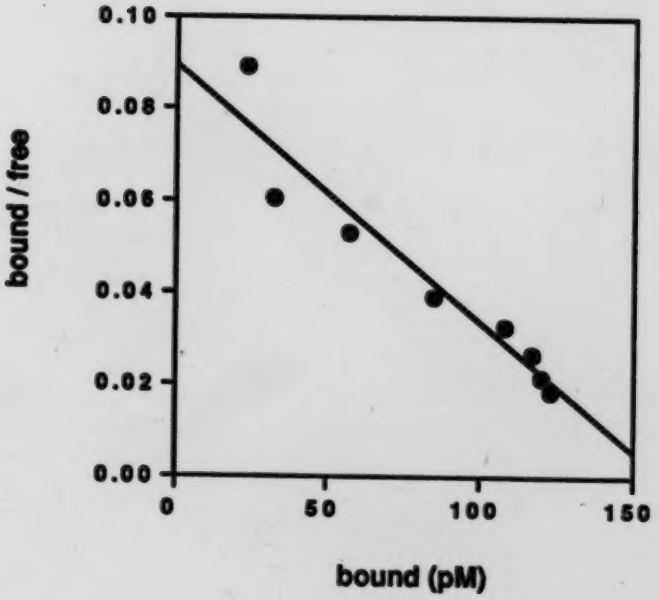
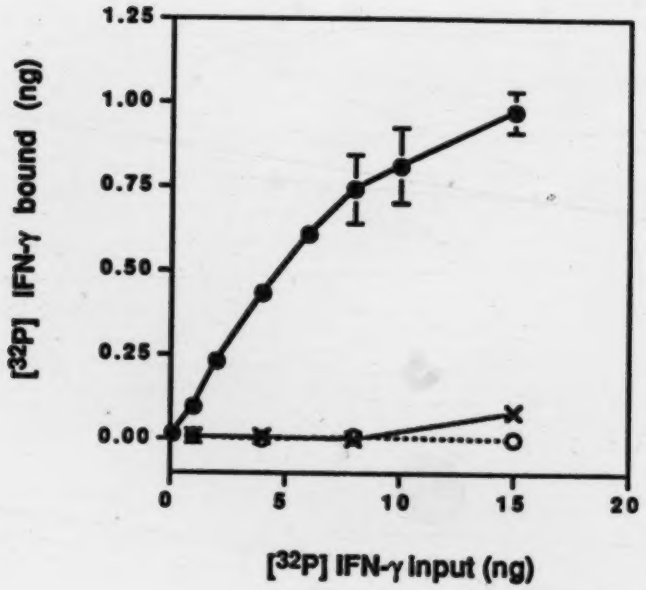
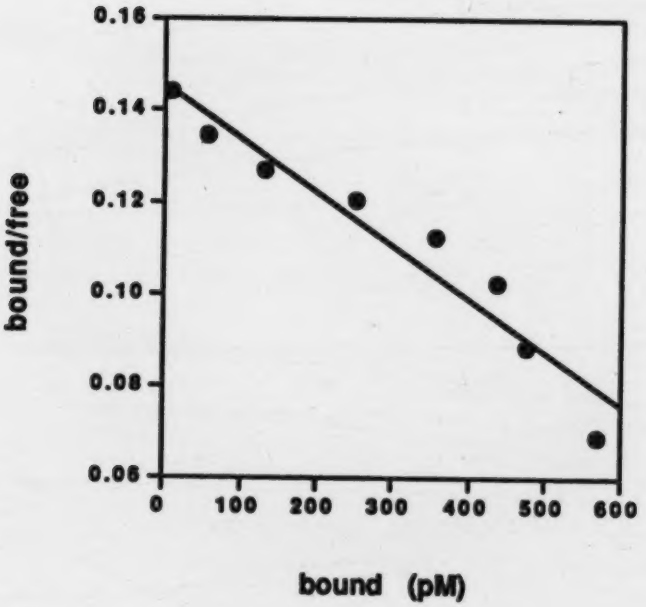

0.10
0.08
0.06
0.04
0.02
0.00
0
50
100
150
bound / free
bound (pM)

Figure III-7. Equilibrium binding analysis of [^{32}P] IFN-γ to the rabbit IFN-γ cellular receptor. (A) solid phase binding analysis of [^{32}P] rabbit IFN-γ (●), [^{32}P] human IFN-γ (○), and [^{32}P] murine IFN-γ (X) to the rabbit IFN-γ receptor on intact rabbit RK13 cells. (B) Scatchard analysis of the binding curve of [^{32}P] rabbit IFN-γ to the RK13 cellular receptor.

A.


1.25
1.00
0.75
0.50
0.25
0.00
[32P] IFN-γ bound (ng)
0
5
10
15
20
[32P] IFN-γ input (ng)

B.


0.16
0.14
0.12
0.10
0.08
0.06
bound/free
0
100
200
300
400
500
600
bound (pM)

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CHAPTER IV

Myxoma Virus M-T7, a Secreted Homolog of the Interferon-γ Receptor, is a Critical Virulence Factor for the Development of Myxomatosis in European Rabbits[1]

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Introduction

Virus survival within immunocompetent hosts requires defensive strategies to circumvent a wide array of potent anti-viral mechanisms (1, 10, 17, 21, 24, 28, 32). Poxviruses have evolved a wide range of immune evasion strategies in order to effectively propagate within their host, including the reduction of inflammation, repression of major histocompatibility class I proteins, inhibition of the complement cascade and interference with cytokines and growth factors (3, 19, 22, 23, 31-33, 35). While the central region of most poxvirus genomes are well conserved and encode for proteins essential for viral replication and structure, the genomic termini are enriched for genes which express proteins that influence virus-host interactions and pathogenesis (3, 34, 35). In order to begin to decipher the complex interplay between host immune defense mechanisms and viral immune evasion strategies, it is useful to consider the unique biological origins of individual poxviruses. Myxoma virus is one of the few poxviruses whose natural host, the South American tapeti rabbit (*Sylvilagus brasiliensis*), has been well established (6, 8, 9, 18, 20). In its natural host, myxoma virus causes benign lesions that persist for many months but are relatively apathogenic, while in the European rabbit (*Oryctolagus cuniculus*), myxoma virus induces a rapidly lethal infection known as myxomatosis (7, 8).

To date, a variety of myxoma proteins have been shown to play a role in myxoma virus pathogenesis in the European rabbit (reviewed in 22, 23). In this communication, we examine the biological role of M-T7, which was originally characterized as a functional homolog of the mammalian cellular IFN-γ receptor (37). M-T7 is expressed from an early viral promoter, processed in the Golgi/endoplasmic reticulum and secreted from myxoma virus infected cells at levels which exceed 10^7 molecules per cell per hour at early times of infection

(Chapter III). Furthermore, M-T7 was shown to bind rabbit IFN-γ with high affinity (K_d=1.2 nM) and efficiently abrogate the antiviral state induced by rabbit IFN-γ on rabbit cells. Unlike the case of IFN-γ receptor homologs encoded by members of the orthopoxvirus genus, myxoma M-T7 protein is specific for the rabbit ligand (Chapters III & V). The open reading frame encoding for M-T7 is present as two copies, one within each of the terminal inverted repeat regions of the myxoma virus genome, where genes important in the determination of virulence are particularly enriched (16, 27, 36).

IFN-γ is a potent anti-viral and immunoregulatory cytokine, and given the importance of IFN-γ in the immune response to poxvirus infection we initiated a study to determine the role of M-T7 during myxoma virus pathogenesis in the European rabbit. Thus, both copies of the M-T7 gene were inactivated with a dominant selectable marker in order to construct a recombinant virus which is deficient for the expression of the M-T7 protein. Here, we compare the disease profile and immune response characteristics in rabbits infected with recombinant M-T7 knockout virus with that of wild-type myxomatosis and speculate that M-T7 may possess biological activities in addition to its previously described biochemical property of interferon-γ inhibition.

Results

Construction and Characterization of M-T7$^-$ Mutant Virus

The myxoma virus M-T7 open reading frame is present as two copies, one within each of the terminal inverted repeats of the 160 kb myxoma virus genome (Figure IV-1a). In order to inactivate both copies of the M-T7 gene, a dominant selection method was adopted, using the *E. coli* guanine phosphoribosyltransferase (Ecogpt) marker, shown to be useful for the construction of other poxviral recombinants (2, 5). A 372 bp SmaI-NruI fragment within the M-T7 coding sequence was removed and replaced with a 950 bp Ecogpt cassette driven by the vaccinia virus 7.5K promoter, creating the recombinant M-T7gpt cassette in pMT7G-4 (outlined in Figure IV-1b). PCR amplification, using primers directed towards the terminal regions of the M-T7 gene, allowed for amplification of M-T7gpt, which was subsequently used to construct the M-T7$^-$ mutant virus, vMyxlac-T7gpt. A recombinant viral stock was prepared by sequential plaque purification under selection pressure, in the presence of mycophenolic acid. As outlined in Chapter II, vMyxlac-T7gpt was subsequently used to create the revertant myxoma virus vMyxT7rev, in which both disrupted copies of the M-T7 gene were fully restored.

To ensure disruption of both copies of the M-T7 open reading frame with M-T7gpt, PCR analysis was performed on DNA isolated from plaque purified virus. As shown in Figure IV-1b, both M-T7 and M-T7gpt contain identical sequences at their terminal ends, to which the M-T7 5' and 3' primers hybridize. Based on size, the parental M-T7 sequence (792 bp) is readily distinguished by agarose gel electrophoresis from the recombinant M-T7gpt (1370 bp) (Figure IV-2a, lanes 1 and 2, respectively). Thus, viral DNA derived from any contaminating wild type vMyxlac or constructs with only one M-T7 allele

disrupted can be distinguished from viral DNA derived from pure recombinant vMyxlac-T7gpt by comparing the size of amplified products of the M-T7 open reading frame using M-T7 primers. After three initial blind passages under mycophenolic acid selection, both wild type and recombinant virus M-T7 gene sequences were clearly present in the uncloned virus stocks, since both species of viral DNA were detected in comparable amounts (Figure IV-2a, lane 3), whereas following three rounds of plaque purification, only recombinant M-T7gpt DNA derived from vMyxlac-T7gpt was detected (Figure IV-2a, lane 4). PCR analysis was also used to confirm the purity of vMyxT7rev stocks (data not shown).

M-T7 was originally characterized as the major secreted protein from myxoma virus infected cells, and was shown to be efficiently expressed at levels capable of neutralizing 2000-3000 units of rabbit IFN-γ per infected cell at early times during infection (ref. 37 and Chapter III) Disruption of both copies of the M-T7 open reading frame, however, completely abrogated expression of detectable M-T7 protein by the recombinant virus vMyxlac-T7gpt, as determined by Western blotting with affinity purified anti-M-T7 antibody (Figure IV-2b, compare lanes 1 and 3). Previously, M-T7 protein was shown to specifically bind and inhibit rabbit IFN-γ (Chapter III). Since vMyxlac-T7gpt does not secrete detectable M-T7 protein, no M-T7-IFNγ complex can be detected either (Figure IV-2b, compare lanes 2 and 4). Furthermore, while supernatants from cells infected with parental myxoma virus efficiently abrogate the antiviral state induced by rabbit IFN-γ on a rabbit cell line, supernatants from cells infected with vMyxlac-T7gpt recombinant virus had no effect on the induction of the antiviral state by rabbit IFN-γ (data not shown). Thus, unlike wild-type myxoma virus which efficiently secretes M-T7 protein that forms inhibitory complexes with

rabbit IFN-γ, the recombinant myxoma virus, vMyxlac-T7gpt, does not express any detectable M-T7 protein.

Effects of M-T7 Disruption on Virus Virulence

No defects were noted in the replication of the M-T7 knockout virus, vMyxlac-T7gpt, as compared with wildtype vMyxlac, in cultured rabbit RK-13 fibroblasts *in vitro* in a single step growth curve analysis (Figure IV-3). Similar growth curves were also measured in primate BGMK cells (data not shown). However, as outlined in Table IV-1, vMyxlac-T7gpt was significantly attenuated following inoculation of European rabbits, as compared to the parental virus, vMyxlac. Dramatic differences were noted in the size and progression of skin lesions, the onset and severity of secondary Gram negative bacterial infections, and the ability of the animal to clear the viral infection. In fact, the majority (12/13) of vMyxlac-T7gpt infected animals exhibited virtually none of the most severe symptoms of myxomatosis that are associated with the wild type virus, but instead recovered completely following a moderated disease course characterized by relatively mild symptoms. Specifically, at four days post-infection, the primary lesions of rabbits infected with vMyxlac-T7gpt were smaller than those characteristic of infection with vMyxlac, and displayed less hemorrhage and necrosis. By seven days post-infection, rabbits infected with the M-T7 knockout virus displayed fewer and smaller secondary lesions (if at all) compared with the numerous, large secondary skin lesions on the ears, eyelids and noses of rabbits following a typical wild-type myxoma infection. Whereas vMyxlac infection typically induces extensive secondary Gram negative bacterial infections (rhinitis and conjunctivitis), only about half of the rabbits infected with vMyxlac-T7gpt showed any symptoms at all of bacterial colonization of the respiratory tract or conjunctiva. Finally, by ten days post-

infection when rabbits infected with vMyxlac possess symptoms of myxomatosis severe enough to require euthanasia, only one of 13 rabbits infected with vMyxlac-T7gpt became moribund. The remaining 12 rabbits ranged in their severity of disease symptoms from extremely mild to moderate, and completely recovered by four weeks. Furthermore, these survivor rabbits were immune to subsequent challenge by wild-type vMyxlac. In a subsequent experiment, two rabbits were infected with vMyxT7rev; one rabbit was sacrificed on day 9 due to severity of symptoms, whereas the second rabbit was sacrificed on day 12 (data not shown). Thus, we conclude that the M-T7 protein plays a significant role in myxoma virus pathogenesis, and can be defined as a classical virus virulence factor.

In the study of other documented virulence factors expressed by the myxoma virus genome, the disease characteristics of vMyxlac have been well characterized (16, 27, 36). In this particular study, three rabbits were injected with vMyxlac for comparative pathological analysis, and six rabbits for histological analysis (described below). Thus, since the construction of vMyxlac in 1991, a minimum of 24 rabbits have now been studied following infection with vMyxlac. While most rabbits injected with vMyxlac became moribund ten days post-infection, typical of the myxomatosis disease progression within this strain, one rabbit was capable of mounting an immune response sufficient for recovery. We have now tentatively assigned a grade II virulence to vMyxlac, using the original nomenclature proposed by Frank Fenner (8).

vMyxlac-T7gpt Exhibits Reduced Viral Dissemination in Infected Rabbits

A common feature of myxomatosis is the rapid and efficient dissemination of virus via lymphatic channels from primary inoculation sites to

numerous secondary sites, most prominently the nose, ears, eyelids, respiratory tract and gonads (8). Our initial observations demonstrated that, while vMyxlac-T7gpt is attenuated in rabbits, the virus is still capable of disseminating to some degree from the primary site of inoculation to the normal spectrum of secondary sites. External examination of rabbits infected with vMyxlac-T7gpt, however, demonstrated a clear reduction in the onset, number and size of lesions at peripheral locations (refer to Table IV-1). It is possible that the $T7^-$ recombinant virus still possesses near normal capacity to disseminate to secondary sites by the infection of macrophages and lymphocytes, but that the cellular immune system has an increased potential to mount a more vigorous and effective response to the infection. Since both the parental virus strain, vMyxlac, and the M-T7 knockout virus each express an *E.coli* lacZ transgene (27), localization of either vMyxlac or vMyxlac-T7gpt virus in infected rabbit tissues is facilitated by staining tissue sections with an antibody against β-galactosidase. Such virus localization studies are important, since a direct correlation between the extent of viral replication and lesion size cannot be assumed. Thus, a time course study was initiated in which six additional rabbits each were infected with either vMyxlac or vMyxlac-T7gpt, and at 4, 7 and 10 days post-inoculation, two rabbits from each group were sacrificed and subjected to a full post-mortem necropsy. Primary and secondary tissue samples were then prepared and immunostained for β-galactosidase as described in Chapter II.

No significant difference in the level of β-galactosidase staining, which corresponds approximately to the level of expression of other myxoma virus late genes, was observed in primary lesions at the inoculation site from vMyxlac or vMyxlac-T7gpt infected rabbits at early stages of infection (data not shown). By 7 days post-inoculation, peripheral tissues from rabbits infected with vMyxlac demonstrated high levels of β-galactosidase, consistent with efficient virus

propagation at secondary sites. Figure IV-4a demonstrates the typical extent of β-galactosidase expressed at day 7 within the secondary ear lesion of a rabbit infected with wild-type virus. While viral propagation was not limited to just this particular section, it does appear to be focused at the epidermis and underlying dermis, although some staining of β-galactosidase is observed throughout the entire dermis. In contrast, a typical section of an ear lesion harvested from a rabbit infected with vMyxlac-T7gpt does not contain any comparable intensely staining areas that would be indicative of unrestrained viral propagation (Figure IV-4b). Similar observations were obtained when secondary site tissue sections were harvested 10 days post-inoculation (not shown). Thus, we conclude that viral load at secondary sites following dissemination was significantly and systematically reduced in rabbits inoculated with the recombinant M-T7$^-$ mutant virus.

vMyxlac and vMyxlac-T7gpt Elicit Distinct Cellular Immune Responses

Since the pathogenic profile of vMyxlac-T7gpt appears to differ significantly from the parent, vMyxlac, with respect to both virulence and ability to establish secondary infection sites, a more detailed histological analysis was performed on the tissues harvested in the time course experiment described above (summarized in Table IV-2). Four days post-inoculation, the primary sites of both vMyxlac and vMyxlac-T7gpt inoculation manifested essentially similar characteristics of disease progression, including edema of the deeper dermis with an increase in the number of mononuclear inflammatory cells diffusely present throughout both the superficial and deeper dermis. While areas which underlay the primary site of vMyxlac inoculation showed an accumulation of inflammatory cells (Figure IV-5a), the primary site of vMyxlac-T7gpt inoculation

appeared to contain a greater level of edema and fewer mononuclear cells in the deeper dermis (Figure IV-5b). No significant differences were observed at any of the external peripheral tissues that normally signal myxomatosis at this time point. A very striking difference was observed, however, between lymph nodes harvested from rabbits infected with each virus. At four days post-inoculation with vMyxlac, draining lymph nodes demonstrated either an absence of, or only a very mild degree of, lymphocyte reactivity in the germinal centers and edema of the sinusoids (Figure IV-6a). By day four, vMyxlac-T7gpt infection, however, induced a dramatic level of lymph node cellular reactivity, characterized by edema of both the subcapsular and medullary sinusoids, an increase in the number of heterophils within the sinusoidal areas, and a reduced population in the central areas of the lymphoid germinal centers with increased mitotic activity (Figure IV-6b). Differences were also observed within another lymphoid organs, namely the spleen. Spleens harvested from animals infected with vMyxlac were essentially indistinguishable from normal uninfected controls, while animals infected with vMyxlac-T7gpt possessed highly reactive and edematous spleens, although not to the levels observed within the peripheral lymph nodes (data not shown). We conclude that by four days post-inoculation animals infected with the wildtype myxoma virus had mounted only a poor cellular immune reaction, if at all, to the infection while in vMyxlac-T7gpt infected rabbits, the cellular reactivity in secondary immune organs was particularly vigorous.

At 7 days post-inoculation, secondary sites of infection, such as the ear and eyelid, were dramatically different between the two viruses (Table IV-2). Animals infected with vMyxlac demonstrated secondary lesions characterized by a thickened and disorganized epidermis with focal erosion and ulceration. Further, a widespread acute inflammatory reaction within the dermis consisted

primarily of heterophils and macrophages, and an acute inflammatory reaction with considerable edema was observed within the subcutis. Secondary lesions from animals infected with vMyxlac-T7gpt, on the other hand, contained a normal epidermis, a focal, mainly mononuclear cell inflammatory reaction with infiltrating lymphocytes predominating within the dermis, and no inflammation but some edema within the subcutis. Thus, in general, while secondary sites of infection with vMyxlac-T7gpt demonstrated what appeared to be a primary inflammatory immune reaction, this reaction was more focalized and did not extend as deeply into the dermis and connective tissues. These results are consistent with our observations at the gross level and suggest that in the absence of T7 expression, reactive lymphocytes now contribute to the clearance of vMyxlac-T7gpt at secondary sites.

Behavior of Reactive Leukocytes at Late Times (day 10) of Infection

By 10 days post-inoculation, the primary site of infection with vMyxlac revealed extensive necrosis of the skin down to the deeper dermis, with an intense cellular inflammatory reaction localized in the deeper dermis immediately superficial to the cutaneous muscle (Table IV-2). The cellular infiltration and accumulation of large numbers of heterophils with smaller numbers of mononuclear type cells occupied approximately one-half of the depth of the deep dermal layer, suggesting a block to the free cellular migration into the primary site of viral replication. Diffuse interstitial inflammation with vascular congestion and an infiltrate of a mixed population of heterophils and lymphocytes accumulated in large masses at the edges of the zone of necrosis. As well, opportunistic coccoid bacteria were colonizing the necrotic epidermal surface. In contrast, while the primary site of infection with vMyxlac-T7gpt also demonstrated full thickness necrosis from the epidermis to the subcutis, a

comparable mass of reactive inflammatory cells within the deeper dermis was not observed. The epidermis was characterized by vacuolation of the epithelial cells with lifting of the necrotic epidermis off the basement membrane with the intervening space being filled with protein rich fluid. As well, the subcutaneous muscle exhibited a diffuse infiltrate of lymphoid cells, with a distinctively lymphoid population developing in the dermis at the edges of the necrotic area. Clear demarcation of the primary site of inoculation was seen, which corresponds to the resolution of these primary lesions observed by day 14 upon gross analysis.

The clustering of inflammatory cells was also more extensive at secondary sites of infection with vMyxlac as opposed to vMyxlac-T7gpt. For example, secondary lesions from the eyelids of rabbits infected with vMyxlac revealed a diffuse inflammatory reaction throughout the full thickness of the connective tissue from the epidermal surface to the conjunctival mucosal surface. Diffuse edema and accumulation of heterophils, mononuclear and lymphoid cells predominated, while the epidermal surface was reactive and appeared somewhat disorganized. In contrast, secondary lesions from the eyelids of rabbits infected with vMyxlac-T7gpt displayed a relatively normal epidermis with an inflammatory reaction, consisting mainly of mononuclear cells with lymphocytes predominating, being restricted to the underlying dermis. Eyelid tissue sections harvested from rabbits infected with vMyxlac and stained with an anti-rabbit CD43 antibody, which recognizes rabbit T lymphocytes, monocytes and macrophages, showed diffuse cellular staining throughout the dermis (Figure IV-7a). However, similar tissue sections taken from rabbits infected with vMyxlac-T7gpt demonstrated a very focal staining pattern of the dermis directly beneath the epidermis (Figure IV-7b). Differences in the organization of the epidermis and dermis can also be seen in these sections.

Thus, in rabbits infected with vMyxlac, an intense, deep and diffuse inflammatory reaction was established, both at the primary site of inoculation, and was particularly prominent beneath sites where secondary lesions form. Importantly, many of the reactive inflammatory cells cluster in large arrays in areas that border the infected site, as if migration of early response inflammatory cells to the infected sites is impeded. In rabbits infected with vMyxlac-T7gpt, however, a less intense yet more focalized cellular immune reaction was observed, primarily at the superficial dermis, as if the reactive leukocytes were now able to more effectively penetrate into the viral lesion to gain increased access to the infected cells. Furthermore, an intense primary cellular immune response was established at early times after infection by vMyxlac-T7gpt within the regional lymph nodes and the spleen, which is not observed to any significant extent upon infection with wild-type myxoma virus.

We conclude that expression of the M-T7 protein in the wild type myxoma infection establishes what appears to be a partial blockade on the effective migration of reactive inflammatory leukocytes into the infected sites, and reduces substantially the extent of communication between sentinel immune cells at the primary site, probably including resident professional antigen presenting cells, and resting lymphocytes in the secondary immune organs. Thus, the loss of M-T7 expression in vMyxlac-T7gpt induces viral attenuation in European rabbits by allowing the development of a more effective cellular immune recognition and subsequent recruitment of activated immune effector lymphocytes from the spleen and lymph nodes to the tissues harboring active virus replication.

Discussion

The importance of IFN-γ in the resolution of poxvirus infections has been well documented (reviewed in 3, 26). For example, recombinant vaccinia virus capable of expressing encoded mouse IFN-γ demonstrates a dramatic attenuation in pathogenicity following infection of immunodeficient mice (14). Similarly, mice treated with a monoclonal antibody directed against mouse IFN-γ during the course of vaccinia virus infection succumb to a lethal infection (29). Using knockout mice that lack the IFN-γ receptor, it was found that IFN-γ increases the susceptibility of mice to vaccinia virus infection by severely diminishing the early defence systems against such an infection, allowing for high viral titres and a lethal pathology (11). Subsequently, IFN-γ was shown to inhibit the replication of vaccinia virus, ectromelia virus, and herpes simplex-1 virus in murine macrophages via the induction of nitric oxide synthase, which enhances macrophage cytotoxicity through the production of nitric oxide (13, 25). Finally, anti-IFN-γ treatment of mice infected with ectromelia virus resulted in enhanced viral spread and replication, and demonstrated that IFN-γ is crucial in all aspects of viral clearance except at the primary site of inoculation (12).

Since IFN-γ is now recognized as one of the most effective host defence mechanisms against viral infection, it is logical to speculate that inhibition of this potent immunoregulatory cytokine would be a critical factor in myxoma virus virulence. Numerous observations lend support to this speculation. First, the most abundantly secreted protein by far from myxoma virus infected cells is the M-T7 protein, which specifically binds rabbit IFN-γ with high affinity (K_d=1.2nM) and can completely block induction of the antiviral state by rabbit IFN-γ (37) and Chapter III) Second, by comparing the pathogenicity of wild-type vMyxlac with an M-T7 knockout virus, vMyxlac-T7gpt, we show here that some of the features predicted to be the consequence of IFN-γ inhibition do indeed appear to play a

role in the progression of a disease state in rabbits infected with myxoma virus. Not only is vMyxlac-T7gpt significantly attenuated in rabbits, but decreased viral dissemination and lower viral titres are observed at secondary sites of infection, while no significant differences in viral replication at primary sites of inoculation are evident. Furthermore, no differences were observed in tissue culture growth curves of vMyxlac and vMyxlac-T7gpt, indicating that M-T7 does not influence the growth of myxoma virus in cultured fibroblasts.

Myxoma virus possesses an extensive and complex arsenal of immune evasion strategies in order to circumvent the host immune defense system and cause a lethal myxomatosis infection (22, 23). M-T7 is thus only one of many proteins shown to date to play a role in myxoma pathogenesis in the European rabbit host. In addition to M-T7, M-T2, MGF, M11L and SERP1 have been identified as myxoma virus virulence factors (reviewed in 23). While the removal of each virulence factor individually attenuates the otherwise lethal myxomatosis disease state, myxoma virus likely requires the entire array of its diverse virulence factors to function in concert in order to completely circumvent the intricate and complex host immune system.

As such, each virulence factor may exercise a distinct activity in modulating different aspects of the potential immune repertoire activated by myxoma virus infection. The data provided here suggest that M-T7 plays a critical role in two distinct, yet likely interlinked, phenotypic responses: (1) blockade of the migration of early response inflammatory cells to the site of viral replication in the infected rabbit dermis, and (2) interruption of the flow of information by resident immune cells which traffic between the site of inoculation and the lymphoid organs where the initiation of acquired immune responses normally develop. The two most dramatic differences in immune responsiveness observed between rabbits infected with vMyxlac and vMyxlac-

T7gpt are the accumulation of inflammatory cells at sites bordering virus replication when T7 protein is expressed, but not when it is deleted, and the high state of lymphocyte reactivity at the lymph nodes and spleen at early times of infection when T7 expression is absent. While only a very mild degree of what we speculate to be antigen specific lymphocyte reactivity is seen within the germinal centers of lymph nodes harvested from vMyxlac infected rabbits, a dramatically elevated level of lymphocyte activation is observed in both lymph nodes and spleen harvested from vMyxlac-T7gpt infected rabbits.

The high degree of reactivity observed within the lymph nodes of vMyxlac-T7gpt infected rabbits substantiates the fact that non-self antigens which enter a host via the skin and mucosa are generally processed and transported by antigen presenting cells (such as macrophages and resident dendritic cells) through the lymphatic system to regional draining lymph nodes, where major histocompatibility complex-restricted activation of both T and B lymphocytes occur. While anti-virus antibodies may help to decrease viral spread, particularly in previously challenged animals, it is believed that virus neutralization through antibody mediated mechanisms is incapable of protecting an immunologically naive host from infection with a rapidly developing virulent poxvirus, such as myxoma. Instead a cell mediated response is believed crucial in determining whether an infection becomes resolved or develops into a lethal syndrome (reviewed in 3). Since the lymph nodes are critical for the initiation of T cell responses to lymph-borne antigens, we propose that myxoma virus M-T7 protein suppresses the antigenic activation of T lymphocytes within these lymphoid tissues, by interrupting some aspect of the communication between antigen presenting cells resident in the infected dermal tissues at the site of inoculation and resting lymphocytes at peripheral nodes. Additionally, in rabbits infected with vMyxlac-T7gpt, not only was virus

propagation at peripheral tissue sites severely inhibited, localization of CD43+ immune cells at both primary and secondary sites was much more highly organized in distinct focal areas beneath the epidermis compared to the wild type infection. Thus, myxoma virus M-T7 protein may not only circumvent the activation of reactive lymphocytes, but histological analysis suggests that it interferes with leukocyte trafficking in general near infected tissues.

Thus, we propose that the differences in viral virulence, viral dissemination and immune response seen with vMyxlac-T7gpt and the wild type virus involve more than the localized inhibition of host IFN-γ. M-T7 was originally characterized as a viral homolog of the mammalian IFN-γ receptor, but since IFN-γ has yet to be shown to influence leukocyte migration, our results indicate that M-T7 may be a multi-functional virulence factor. Indeed, there are precedents for viral proteins which are capable of performing more than one anti-immune activity. Tanapox virus, for example, secretes a glycoprotein at early times of infection which is capable of binding and inhibiting three completely distinct cytokines, namely human IFN-γ, IL-2 and IL-5 (4). Recently, we have observed that M-T7, in addition to its ability to bind and inhibit rabbit IFN-γ, is capable of binding a wide array of chemotactic cytokines collectively referred to as chemokines (15). Although it is not yet clear if the chemokine binding activity of M-T7 contributes to the apparently aberrant migration of leukocytes in the vicinity of myxoma lesions, mutational analysis of M-T7 may provide clues as to the functional domains of this protein which are important to virulence. Interestingly, sequence alignment of the soluble poxviral IFN-γ receptor homologs with the known cellular mammalian IFN-γ receptors (Chapter III) demonstrated that all of the known poxviral proteins contain approximately 60 amino acids at their carboxy terminal ends which are unrelated to the mammalian receptors, but are well conserved among each other. We are

currently investigating the role this carboxy terminal region plays in the structure and function of the M-T7 protein.

In order to circumvent host anti-viral immune mechanisms and establish a lethal myxomatosis disease state, myxoma virus elicits a diverse array of virulent factors, each of which plays a define yet co-ordinated role within the viral infection. M-T7 is a critical component of this immune evasion strategy and the dissection of the apparently multiple roles played by this protein, which may not be limited to just IFN-γ inhibition, becomes exceedingly relevant to the identification of new host molecules which orchestrate the host immune response to virus infections.

Table IV-1: Pathogenicity of vMyxlac-T7gpt in European rabbits

Day	vMyxlac (wild type)	vMyxlac-T7gpt (T7 mutant)
0	Inoculation of rabbits with vMyxlac[a]	13 rabbits inoculated intradermally at 2 sites with 1000 pfu vMyxlac-T7gpt per site
4	Primary lesions at inoculation sites, approx. 2cm, soft, flat, turning necrotic in center	Primary lesions at inoculation sites, approx. 1cm, raised, hard; slight necrosis detected in lesions of 2 rabbits only
7	Gram negative bacterial infections of nasal and conjunctival mucosa; multiple secondary skin lesions on face and ears	Minor Gram negative infections of nasal and/or conjunctival mucosa in 6 of 13 rabbits; secondary skin lesions on face and ears smaller than observed with vMyxlac (wild type)
10	Severe Gram negative bacterial infections in conjunctiva and respiratory tract; secondary skin lesions turning necrotic; rabbits sacrificed due to increasing severity of symptoms	Gram negative infections undetected in 5 of 13 rabbits, mild in 4 of 13 rabbits, severe in 4 of 13 rabbits; secondary lesions numerous but small; 12 of 13 rabbits highly responsive to stimuli; 1 rabbit sacrificed due to severity of symptoms
14	---	Gram negative infections mild to absent; primary and secondary lesions regressing
28	---	Absence of Gram negative infections; primary lesions consist of small, dry scabs; small scars remain from secondary lesions
		Surviving rabbits immune to subsequent challenge with vMyxlac (wild type) infection

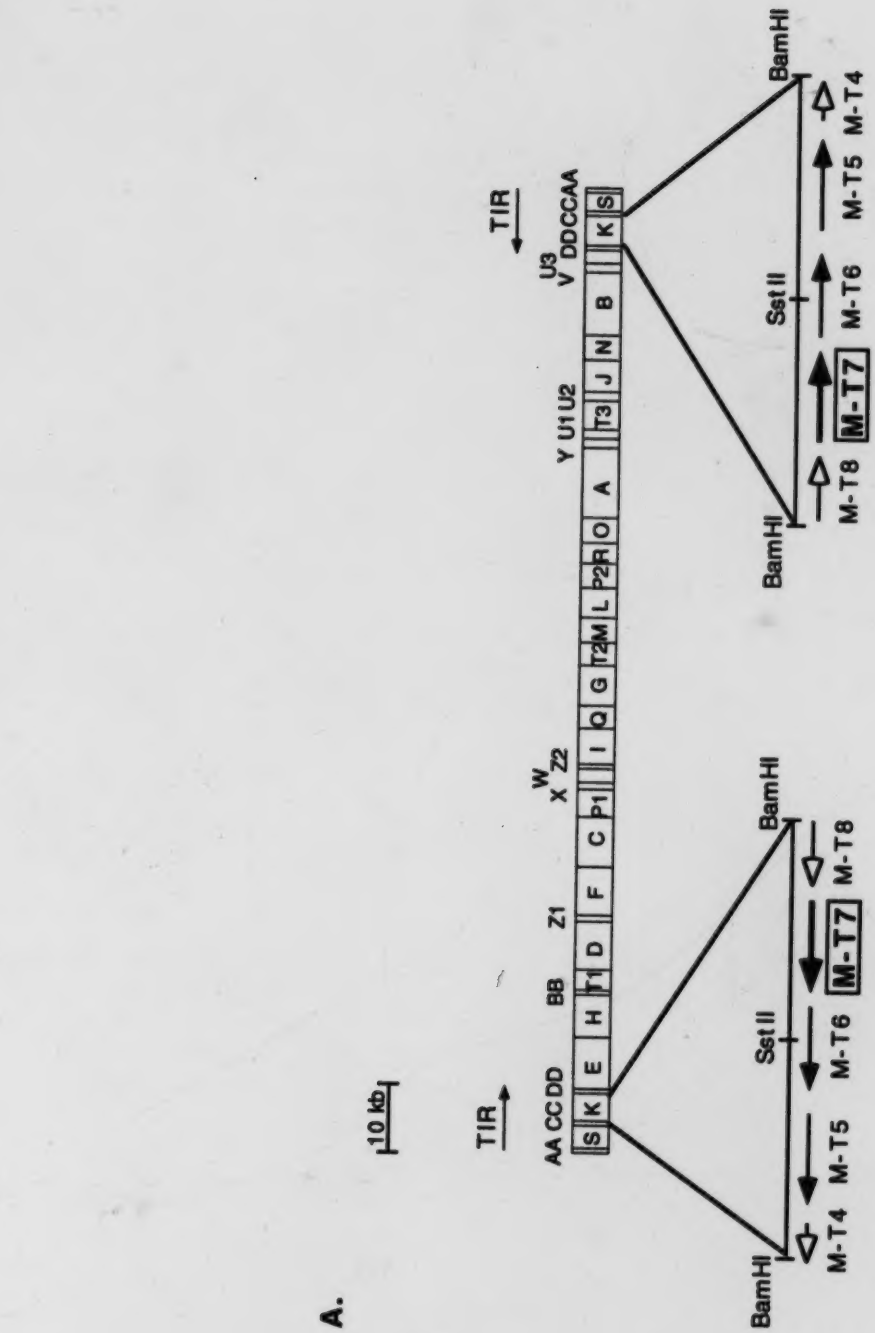
[a]As described in Materials and Methods, the pathogenic profile is a summation of multiple studies carried out with vMyxlac, which carries a lacZ cassette at an intergenic location in myxoma, strain Lausanne.

Table IV-2: Major histological differences between wild type myxoma and vMyxlac-T7gpt infection in European rabbits

	vMyxlac (wild-type)	vMyxlac-T7gpt (M-T7 mutant)
Day 4	Primary sites[a] •acute inflammatory reaction, primarily heterophils and macrophages, mostly focalized in subdermal tissues below areas of viral replication •edema	Primary sites[a] •less inflammatory reaction, primarily heterophils and macrophages, dispersed throughout lesion •enhanced edema
	Secondary sites[b] •no detectable reaction	Secondary sites[b] •no detectable reaction
	Lymph nodes- very mild activation	Lymph nodes-highly reactive lymphocytes
	Spleen-normal	Spleen-reactive lymphocytes
Day 7	Primary sites[a] •acute coagulation necrosis of epidermis and dermis •acute inflammation with edema •hemorrhage	Primary sites[a] •acute coagulation necrosis of epidermis and dermis •acute inflammation with edema •hemorrhage
	Secondary sites[b] •thickened, disorganized epidermis •acute inflammatory reaction in dermis and subcutis •heterophils and macrophages predominate	Secondary sites[b] •normal epidermis •mild inflammatory reaction in dermis, some edema in subcutis •infiltrating lymphocytes predominate
Day 10	Primary sites[a] •full thickness necrosis of dermis •intense inflammatory reaction in deep dermis •heterophils predominate •degeneration of epithelium	Primary sites[a] •full thickness necrosis of dermis •no inflammatory reaction in deep dermis •infiltrating lymphocytes predominate •vacuolization of epithelium
	Seconday sites[b] •thickened, disorganzied epidermis •intense inflammation with edema •heterophils and macrophages predominate	Seconday sites[b] •normal epidermis •mild inflammation with edema •infiltrating lymphocytes predominate

[a]primary site samples taken from site of inoculation in the thigh
[b]secondary site samples taken from ear, lip, nose and eyelid

Figure IV-1. Construction of a recombinant myxoma virus containing a disrupted M-T7 gene. (A) Location of M-T7 open reading frame within the 160 kb myxoma virus genome. The BamHI map of the myxoma virus DNA genome (30) is illustrated. The M-T7 open reading frame exists in two copies within the BamHI K fragment, located within the terminal inverted repeat region of the myxoma virus genome. Location of the single SstII site within the BamHI K fragment is illustrated. Filled arrows denote complete open reading frames within the BamHI K fragment, whereas open arrows indicate flanking open reading frames that span the BamHI sites within this fragment. (B) Construction of vMyxlac-T7gpt disruption virus. An SstII-BamHI fragment, containing the entire M-T7 open reading frame, was cloned into pBluescript, creating the plasmid pKMBD-8. A 372 bp internal SmaI/NruI fragment of the M-T7 coding sequence was removed, and upon addition of BglII linkers, replaced with a 950 bp p7.5Ecogpt BglII cassette, creating the plasmid pMT7G-4. PCR amplification using primers directed towards the 5' and 3' ends of the M-T7 gene was used to obtain 1370bp of M-T7gpt DNA. M-T7gpt DNA was subsequently transfected into vMyxlac infected cells to create the recombinant virus vMyxlac-T7gpt. The size of the M-T7 gene of wild-type vMyxlac, as amplified with the primer pairs used to create M-T7gpt, is illustrated for comparison.


A.
10 kb
TIR
TIR
AA CC DD
BB
Z1
W
X
Z2
Y
U1 U2
U3
V
DD CC AA
S K E H T1 D F C P1 I Q G T2 M L P2 R O A T3 J N B S K
BamHI
Sst II
BamHI
M-T4 M-T5 M-T6 M-T7 M-T8
BamHI
Sst II
BamHI
M-T8 M-T7 M-T6 M-T5 M-T4

B.

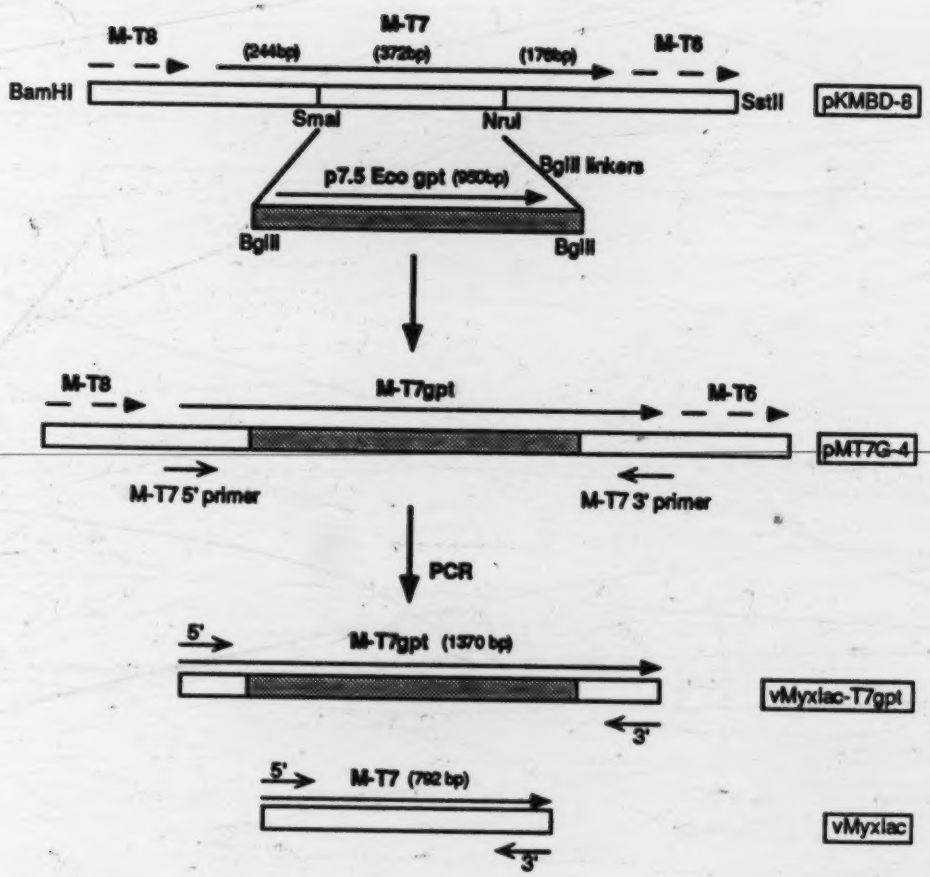
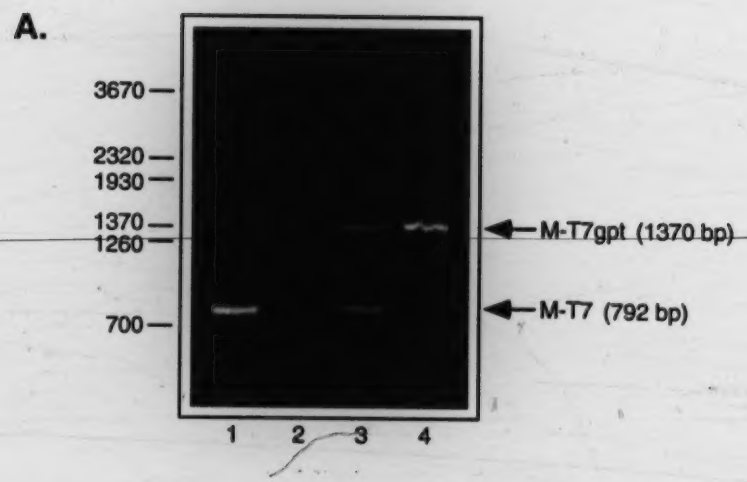

M-T8
M-T7
(244bp)
(372bp)
(176bp)
M-T6
BamHI
SmaI
NruI
SstII
pKMBD-8
p7.5 Eco gpt (960bp)
BglII linkers
BglII
BglII
M-T8
M-T7gpt
M-T6
pMT7G-4
M-T7 5' primer
M-T7 3' primer
PCR
5'
M-T7gpt (1370 bp)
vMyxlac-T7gpt
3'
5'
M-T7 (792 bp)
vMyxlac
3'

Figure IV-2. Characterization of M-T7 from wild-type vMyxlac and recombinant vMyxlac-T7gpt viruses. (A) Agarose gel electrophoresis of PCR products amplified by M-T7 5' and 3' primers (see Figure 1A). Migration of wild-type M-T7 (lane 1) and recombinant M-T7gpt (lane 2) amplified from pKMBD-8 and pMT7G-4 plasmid DNA, respectively. PCR analysis of viral DNA following three blind passages under mycophenolic acid selection pressure illustrates the presence of both M-T7 (792 bp) and M-T7gpt (1370 bp) DNA (lane 3). Following three rounds of plaque purification from a single viral focus, a purified stock of vMyxlac-T7gpt virus was analyzed (lane 4). Location of BstEII cut λ markers is shown on the left. (B) Detection of M-T7 protein secreted from virus infected cells by Western blotting analysis. Supernatants from vMyxlac-T7gpt infected cells (lane 1) and vMyxlac infected cells (lane 3), subjected to Western blotting analysis using affinity purified anti-M-T7 (E266) antibody. M-T7-IFNγ complex is measured following chemical crosslinking of rabbit IFNγ to supernatants from vMyxlac-T7gpt infected cells (lane 2) and vMyxlac infected cells (lane 4). The location of low molecular weight markers is shown on the left.


A.
3670
2320
1930
1370
1260
700
M-T7gpt (1370 bp)
M-T7 (792 bp)
1
2
3
4

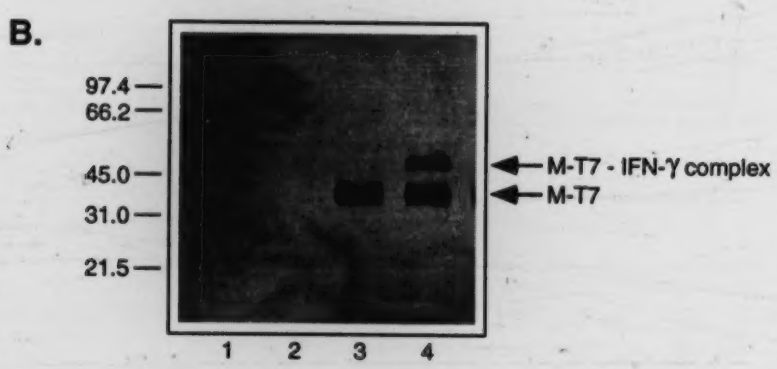

B.
97.4
66.2
45.0
31.0
21.5
M-T7 - IFN-γ complex
M-T7
1
2
3
4

Figure IV-3. Single step growth analysis of the replication of vMyxlac and vMyxlac-T7gpt in cultured rabbit kidney fibroblasts. RK13 cells were infected with vMyxlac (filled squares) or vMyxlac-T7gpt (open squares) with a multiplicity of infection of 5. Cultures were harvested at various times post inoculation and infectious virus titered on RK13 cells. Each viral growth assay was performed in triplicate.

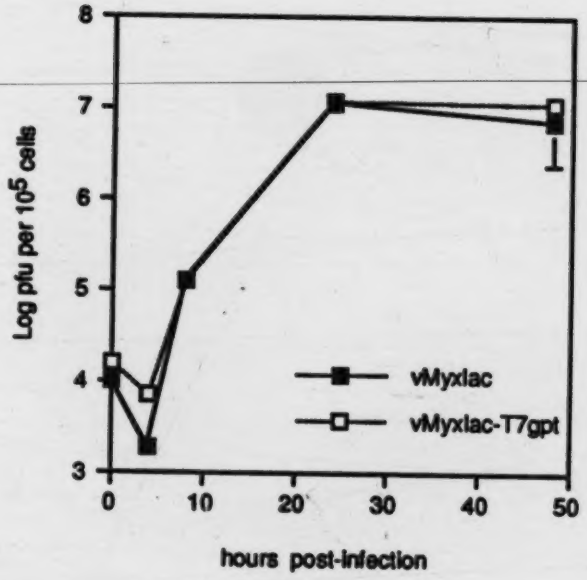
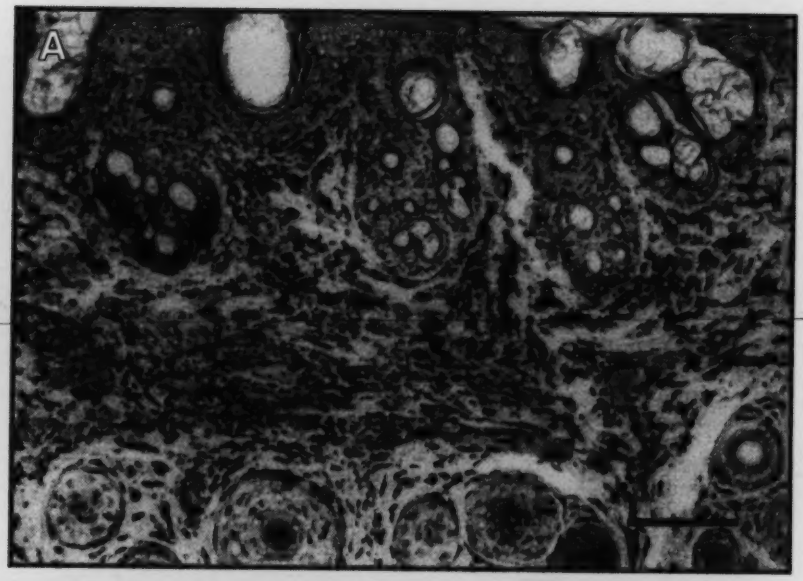

8
7
6
5
4
3
0
10
20
30
40
50
Log pfu per 10^5 cells
hours post-infection
vMyxlac
vMyxlac-T7gpt

Figure IV-4. Secondary ear lesions harvested at Day 7 post-inoculation and stained with an anti-β-galactosidase antibody. (A) Ear lesion from a rabbit infected with wild-type vMyxlac virus. High levels of β-galactosidase staining correlates with the extent of virus late gene expression. (B) Ear lesion from a rabbit infected with recombinant vMyxlac-T7gpt virus. Tissue sections were harvested and stained as outlined in Chapter II. 100μm scale is shown. Immunohistological staining performed by M. Garbutt.


A

B

Figure IV-5. Primary site of infection tissue harvested at Day 4 post-inoculation. (A) Primary site of vMyxlac inoculation possesses an enhancement of accumulated inflammatory cells within the deeper dermis (indicated by arrows). (B) Primary site of vMyxlac-T7gpt inoculation demonstrates enhanced edema and lack of inflammatory cell accumulation within the deeper dermis, as compared to the wild type lesion in panel A. 1mm scale is shown. Histological analyses performed by Dr. P. Nation.

A

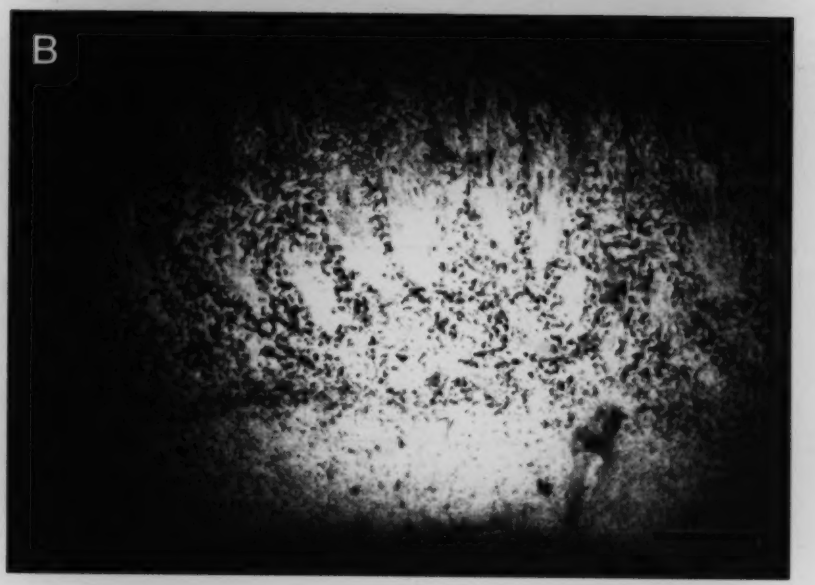
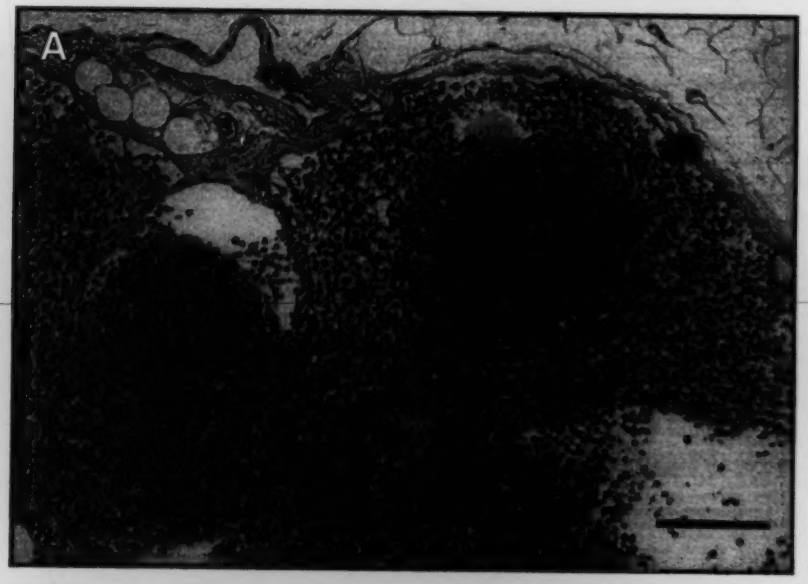

B

Figure IV-6. Lymph node tissue harvested at Day 4 post-inoculation. (A) Lymph node tissue from a rabbit infected with vMyxlac possesses low level of cellular activity within the germinal centers. (B) Highly reactive lymph node harvested from a rabbit infected with vMyxlac-T7gpt. Note more ordered structure and greater cellularity of germinal centers. Arrows indicate zone of increased mitotic activity within the lymphoid germinal centers. Tissue sections were harvested and stained as outlined in Chapter II. 100μm scale is shown. Histological analyses performed by Dr. P. Nation.


A

B

Figure IV-7. Secondary eyelid lesions harvested at Day 10 post-inoculation and stained with an anti-CD43 antibody to identify rabbit T cells, monocytes and macrophages. (A) Tissue section from a rabbit lesion infected with vMyxlac demonstrates extensive diffuse staining of CD43 positive cells throughout the dermis. (B) CD43 positive cells are highly focalized within the superficial dermis in lesions from a rabbit infected with vMyxlac-T7gpt. Tissue sections were harvested and stained as outlined in Chapter II. 100μm scale is shown. Immunohistological staining performed by M. Garbutt.

A

B

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CHAPTER V

Species Specificity of Ectromelia Virus and Vaccinia Virus Interferon-γ Binding Proteins[1]

[1]All data herein have been published: Mossman, K., Upton, C., Buller, R.M.L., McFadden, G. (1995) *Virology* **208:** 762-769.

Introduction

IFN-γ is a pleiotropic cytokine which serves critical functions within the immune system both in the presence and absence of pathogenic challenge (7, 8, 26). The importance of IFN-γ in the response to viral infection has been amply demonstrated, particularly in the case of poxviruses (3, 12, 17). In a murine experimental model, infection with the *Orthopoxvirus* vaccinia virus, which is effectively cleared by the immune system, becomes lethal upon addition of monoclonal antibodies to IFN-γ (22). Likewise, in athymic nude mice, where vaccinia virus infection is lethal, clearance is attained when infection with a recombinant virus expressing IFN-γ is utilized (14, 20). IFN-γ has also proved critical in the recovery of mice from ectromelia virus, the causative agent of mousepox (12).

In response to the potent anti-viral and immunoregulatory effects elaborated by IFN-γ during viral infection, poxviruses have evolved a variety of defence mechanisms to effectively reduce the efficiency of the host immune response. For example, IFN-γ (and especially IFN-α and IFN-β) induction of anti-viral activity through the RNA-dependent protein kinase (PKR) pathway is blocked at the stage of double-stranded RNA activation and phosphorylation of the initiation factor eIF2-α by vaccinia virus E3L and K3L intracellular proteins, respectively (4, 5). While E3L and K3L block certain intracellular IFN-γ induced pathways, poxviruses are also capable of inhibiting IFN-γ extracellularly, prior to receptor engagement. Cells infected by the *Leporipoxvirus* myxoma virus were shown to secrete a 37 kDa protein, designated M-T7, which possesses homology to the ligand binding domain of the known mammalian IFN-γRs, and effectively functions as a soluble IFN-γR homolog (25). Here, we demonstrate that secretion of an IFN-γ binding protein is not unique to myxoma virus, and

investigate the properties of IFN-γ binding proteins from ectromelia virus and vaccinia virus.

Results

Many Poxviruses Express Soluble IFN-γR Homologs

Due to the accumulation of genomic sequence information, putative IFN-γR homologs have recently been discovered to be encoded by a variety of poxviruses (reviewed in 18). Figure V-1 demonstrates the presence of complexes containing radiolabeled rabbit IFN-γ bound to secreted proteins from the supernatants of cells infected with myxoma, SFV, vaccinia (strains WR and IHDW), ectromelia, cowpox and rabbitpox viruses (Figure V-1 lanes 2-8, respectively), which are not seen in mock infected supernatants (Figure V-1, lane 1). In subsequent studies, all vaccinia virus strains tested were found to possess a comparable IFN-γ binding protein (2). In the crosslinking assays shown in Figure V-1, monomer, dimer and higher molecular weight complexes of IFN-γ are detected. Frequently, the intensity of dimeric IFN-γ decreases upon the formation of IFN-γ/IFN-γR homolog complexes, which might suggest that the IFN-γ binding proteins are preferentially disrupting IFN-γ dimers. Crosslinking assays, while useful for demonstrating protein associations, do not necessarily reflect all of the biological interactions between protein species. Thus, the decrease in intensity of dimeric IFN-γ observed in Figure V-1 does not formally prove that the ligand dimer is the preferential substrate for the formation of IFN-γ/IFN-γR homolog complexes. The issue of monomeric versus dimeric IFN-γ specificity for the poxviral IFN-γR homologs remains to be addressed. The sizes of the shifted complexes are consistent with the predicted molecular mass of the known viral proteins complexed to the IFN-γ ligand, taking into consideration post-translational modifications such as glycosylation, which could account for some of the minor bands observed. The myxoma virus M-T7 protein, the most abundantly secreted protein from myxoma virus infected cells, demonstrated by

the intensity of the labeled complex, is known to be N-glycosylated (data not shown). Thus, it appears that the expression of a soluble IFN-γ receptor homolog is a common feature used by poxviruses to counteract the pleiotropic effects of IFN-γ.

Characterization of the Ectromelia Virus IFN-γR Homolog

Since IFN-γ was found to be essential for the clearance of ectromelia virus in a murine model (12), we sought to further characterize the ectromelia virus IFN-γ binding protein observed in Figure V-1. DNA sequencing of the ectromelia virus genome revealed the presence of a complete open reading frame related to the myxoma virus M-T7 gene and the vaccinia virus B8R gene (Figure V-2A). The DNA sequence 5' of the ectromelia virus open reading frame is consistent with transcription at early times post-infection (6) and an early transcription termination signal (TTTTTAT) is present 30 nucleotides 3' of the open reading frame. As expected, the ectromelia virus IFN-γ binding protein is most closely related to the homologs from vaccinia and variola viruses (Figure V-2B) since all three are orthopoxviruses, whereas myxoma virus belongs to the leporipoxviridae. While the poxviral IFN-γ binding proteins contain relatively low homology (~20%) to the ligand binding domain of the human and murine IFN-γ receptors, high homology is observed within the orthopox and leporipox genera (Chapter III). The length of these proteins are very similar and the number of amino acid differences between the ectromelia-vaccinia, ectromelia-variola and vaccinia-variola viral pairs are 19, 23 and 18, respectively. In particular, while the myxoma virus M-T7 protein contains all eight conserved cysteines believed to be important for the structure of the ligand binding domain (Chapter III), only six of the eight cysteines are conserved in

each of the orthopoxviruses, with the first two cysteines replaced by tyrosines in each case.

Species Specificity of Ectromelia Virus IFN-γ Binding Protein

Unlike the myxoma virus M-T7 protein, which is specific for binding rabbit IFN-γ (Chapter III), the ectromelia virus IFN-γ binding protein does not exhibit comparable species specificity (illustrated in Figure V-3). The IFN-γ binding protein present in ectromelia virus infected supernatants is capable of binding rabbit, human and murine IFN-γ (lanes 1, 3 and 5, respectively), while no binding with these three ligands is seen in mock infected supernatants (lanes 2, 4, and 6, respectively). The relative intensities of the shifted complexes might suggest that the ectromelia virus IFN-γ binding protein preferentially binds rabbit IFN-γ, but consideration must be given to the number of phosphorylation sites (rabbit and human IFN-γ have two sites, murine IFN-γ has one), and to the differential radiolabeling at these sites, which is reflected by the monomeric IFN-γ levels.

To further characterize the species specificity of the ectromelia virus IFN-γ binding protein, chemical cross-linking assays involving cross-species competition were performed. Supernatants from ectromelia virus infected cells were incubated with [^{32}P]-human, -murine, and -rabbit IFN-γ, either alone or in the presence of increasing amounts of unlabeled competitor IFN-γ. As seen in Figure V-4a, the ectromelia virus IFN-γ binding protein efficiently binds [^{32}P]-human IFN-γ (lane 1), consistent with previous results (Figure V-3), and the labeled human IFN-γ is comparably competed from the complex by unlabeled rabbit, human and murine IFN-γ (lanes 2-13). In all cases, complete competition is observed with a 40-fold excess of unlabeled IFN-γ (lanes 5, 9 and 13). Similar results are seen when [^{32}P]-murine IFN-γ (Figure V-4b) or [^{32}P]-rabbit

IFN-γ (Figure V-4c) are used as the radiolabeled ligand. Thus, it appears that the ectromelia virus IFN-γ binding protein has similar affinities for human, murine and rabbit IFN-γ.

Species Specificity of the Vaccinia Virus IFN-γ Binding Protein

Since vaccinia virus also expresses an IFN-γ binding protein (Figure V-1, lanes 4 and 5), it was of interest to determine the specificity of this protein, using the same cross-species competition cross-linking assay described above. Like the ectromelia virus IFN-γ binding protein, the vaccinia virus homolog is capable of binding radiolabeled human, murine and rabbit IFN-γ (lane 1 in Figure V-5a-c, respectively). However, the vaccinia virus IFN-γ binding protein does not have the same relative affinity for all three ligand species. As seen in Figure V-5a, excess unlabeled rabbit IFN-γ (lanes 2-5) and human IFN-γ (lanes 6-9) successfully compete away the labeled human ligand from the complex, whereas excess unlabeled murine IFN-γ (lanes 10-13) is unable to function as a competitor, even at a 40-fold excess over the labeled human ligand (lane 13). Similar results are seen if rabbit IFN-γ replaces human IFN-γ as the radiolabeled ligand (Figure V-5c). Accordingly, excess unlabeled rabbit and human IFN-γ successfully compete away [^{32}P]-murine IFN-γ from the labeled complex (Figure V-5b). Complete competition is seen with a 10-fold excess of rabbit and human IFN-γ (lanes 3 and 7, respectively), while a 40-fold excess of unlabeled murine IFN-γ is necessary to compete with its labeled counterpart to the vaccinia virus IFN-γ binding protein (lane 13). Thus, the putative vaccinia virus IFN-γR homolog has reduced relative affinity for murine IFN-γ, as compared to the affinity for either rabbit or human IFN-γ.

Binding of IFN-γ is Specific to Secreted Viral Proteins

Soluble forms of mammalian IFN-γ cellular receptors are known to be shed from cells (19, 21), and thus the question arises of whether the detected soluble IFN-γ binding proteins are virus encoded. It is conceivable that soluble forms of the cellular receptor could be shed as a result of viral infection, for example by increased cell surface proteolysis, and thus potentially the binding observed in the crosslinking assays might not be specific to virally secreted proteins. Since all of the viral supernatants were cultured from monkey kidney cell lines, it was important to ascertain the species specific binding properties of monkey kidney cellular receptors. As seen in Figure V-6, monolayers of BGMK cells are capable of binding human and rabbit IFN-γ, but not murine IFN-γ. Since both ectromelia virus and vaccinia virus are capable of binding murine IFN-γ, albeit with different affinities, shed cellular receptors cannot be responsible for the binding of IFN-γ observed in poxvirus infected supernatants. Furthermore, the migration of the shifted complexes observed in Figure V-1 vary with the different viruses, which is inconsistent with shed cellular receptors being solely responsible for IFN-γ binding. Although one cannot formally rule out the possibility that viral infection does indeed cause shedding of cellular receptors, and that a portion of the binding observed is due to these shed receptors, at least in the case of myxoma virus, disruption of the M-T7 gene results in complete loss of IFN-γ binding proteins in the supernatant of virus-infected cells (Chapter IV).

Discussion

For successful propagation and survival, poxviruses have evolved numerous strategies to circumvent the host immune system (for reviews see 3, 16, 23, 24). IFN-γ is a critical regulator of the immune system, and the importance of this pleiotropic cytokine in the immune response to poxvirus infection has been clearly demonstrated (3, 12-14, 17, 20, 22). As such, mechanisms to combat the potent anti-viral effects of IFN-γ would certainly be advantageous to poxviruses. While the myxoma virus M-T7 protein is the first *bona fide* IFN-γR homolog shown to actually inhibit IFN-γ, comprehensive sequencing analysis has suggested the presence of IFN-γ binding proteins in the genomes of SFV, vaccinia virus, variola virus and swinepox virus (Chapter III).

In this report, we demonstrate that myxoma virus is not unique in elaborating an IFN-γR homolog, and that the ability to combat the immunomodulatory effects of IFN-γ by secreting an IFN-γ binding protein appears to be a characteristic conserved among many, if not all, poxviruses. Crosslinking assays using radiolabeled rabbit IFN-γ and supernatants from a variety of poxviral infected cells illustrated that all of the poxviruses tested express an IFN-γ binding protein, similar in molecular mass to the 37 kDa myxoma virus M-T7 protein. Since the clearance of ectromelia virus within a murine model was found to be dependent on the presence of IFN-γ (12), expression of an IFN-γ binding protein by ectromelia virus and discovery of the corresponding open reading frame was not surprising. Although studies of mammalian soluble IFN-γRs have characterized physiological complexes to consist of two soluble receptors to one IFN-γ homodimer (11), it must be emphasized that while crosslinking competition assays are useful for indicating

the hierarchy of binding affinities, they are unable to quantitate the stoichiometry of interaction. Further experiments are currently in progress to address this question.

The issue of ligand specificity has relevance to virus evolutionary history. Myxoma virus, which has evolved in the tapeti, or South American brush rabbit (9), demonstrates strict species specificity for binding and inhibiting rabbit IFN-γ, and possesses no measurable affinity for either human or murine IFN-γ (Chapter III). As such, the myxoma virus M-T7 protein is similar to the mammalian cellular receptor complexes, which demonstrate specificity in binding their cognate ligands (1, 10). Since myxoma virus is one of the few poxviruses for which an evolutionary origin is firmly established, it is feasible to speculate that interactions involving poxviral binding proteins, such as the IFN-γR homologs, may lend insight into the evolutionary origin of other poxviruses. In light of both the species specific nature of the myxoma virus M-T7 protein for the rabbit IFN-γ ligand, and the necessity of IFN-γ in the clearance of an ectromelia virus infection within a murine model, it was surprising to demonstrate that the ectromelia virus IFN-γR homolog possesses the same relative affinity for all three ligands tested. The question is thus raised concerning the evolutionary origin of ectromelia virus. Potentially, either ectromelia virus has been passaged within a series of vertebrate hosts, thus obtaining comparable affinity for a variety of ligand species, or its evolutionary host is equally diverged from rabbits, mice and humans.

Vaccinia virus, widely used as a vaccine against variola virus, the causative agent of smallpox, is a prominent poxvirus whose natural history remains obscure. Thus it is important to ascertain the specificity of the vaccinia virus IFN-γR homolog, in an attempt to clarify the evolutionary history of this virus. Due to the high degree of homology between the three known

orthopoxvirus IFN-γ binding proteins, it was of interest to discover a significant difference in the relative affinity of binding to murine IFN-γ between the vaccinia virus and ectromelia virus IFN-γR homologs. In independent studies, it has been shown that the vaccinia virus IFN-γ binding protein from strains other than the WR and IHDW strains tested here do not significantly interact with murine IFN-γ (2). Thus, it appears that the evolution of vaccinia virus is not as obvious as that of myxoma virus, and identification of likely vertebrate hosts of the progenitor of vaccinia virus must await a survey of other mammalian IFN-γ ligands and analysis of the relevant dissociation constants, using purified viral receptor homologs.

The comparative study of members of the poxvirus family is an important endeavor as it contributes to our understanding of the evolution of the molecular basis of virus virulence. Over millions of years of evolution, these viruses have flourished by establishing stable relationships with their hosts, in part by elaborating a plethora of anti-immune strategies in order to minimize immune recognition and clearance. One such strategy is the production of soluble IFN-γR homologs (25), which counteract the potent and pleiotropic effects of IFN-γ, a cytokine which has been shown to be most important in the recovery of the mouse from infections with the natural viral pathogens, such as murine cytomegalovirus (15) and ectromelia virus (12). Although the systemic recovery from ectromelia virus infection was dependent in part on the ability of IFN-γ to induce the enzyme nitric oxide synthase, responsible for generation of nitric oxide, a potent anti-viral molecule (13), IFN-γ has apparently little anti-viral effect at the primary infection and transmission site in the foot-pad (12, 15). Conceivably, in the first 24 hrs of the infection, the rate of production of ectromelia virus soluble IFN-γR homolog in this peripheral site of the skin is

sufficient to neutralize the endogenously produced IFN-γ, thereby attenuating and delaying the acute inflammatory response.

Poxviruses constitute a potentially valuable repository of both cytokine homologs and cytokine inhibitor homologs (18, 23, 24). However, in order to fully comprehend the role(s) these inhibitors play, a further appreciation of the evolutionary origins of these viruses becomes exceedingly important. Fortunately, the poxvirus system is an attractive one in which to study such virus-host interactions in order to address this issue.

Figure V-1. Poxviruses express secreted IFN-γ binding proteins. Supernatants from mock, myxoma virus (MYX), Shope fibroma virus (SFV), vaccinia virus (VV, strains WR and IHDW), ectromelia virus (EV), cowpox virus (CPV), and rabbitpox virus (RPV) infected cells were chemically cross-linked to [^{32}P]-rabbit IFN-γ. Location of monomer and dimer forms of IFN-γ are indicated with arrows, as is the labeled complex between the poxviral IFN-γ binding proteins and radiolabeled rabbit IFN-γ. Molecular weight marker sizes are indicated on the left.

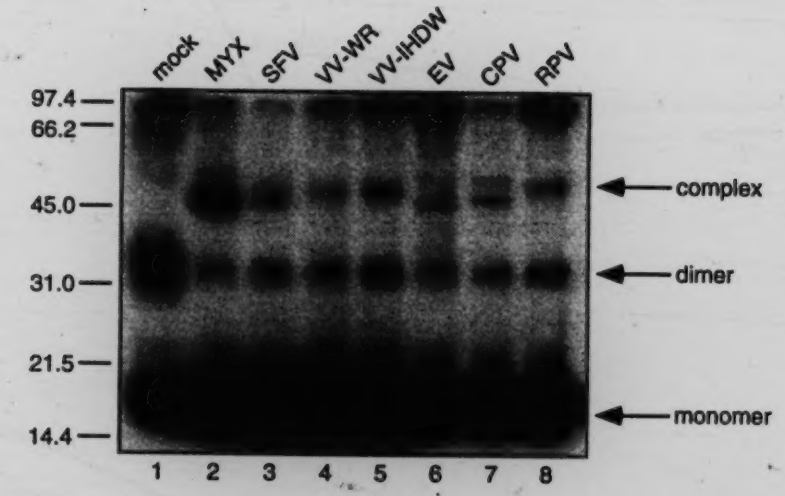
mock
MYX
SFV
VV-WR
VV-IHDW
EV
CPV
RPV
97.4
66.2
45.0
31.0
21.5
14.4
complex
dimer
monomer
1 2 3 4 5 6 7 8

Figure V-2. Ectromelia virus IFN-γ receptor homolog. (A) DNA sequence and translation of a region encompassing the ectromelia virus IFN-γ receptor homolog gene. The partial upstream ORF is homologous to vaccinia virus B7R of vaccinia virus (strain Copenhagen). Stars indicate the location of stop codons. (B) Amino acid alignment of IFN-γ receptor homologs encoded by ectromelia virus, vaccinia virus (VV, strain Copenhagen) and variola virus (VAR, strain India1967). Conserved cysteines believed to be involved in formation of stabilizing disulfide bonds are boxed. Closed circles denote the location and number of amino acid changes between the three peptide sequences. The gap introduced to provide maximal alignment is indicated with a dot.

A.

```
   1  AAAGGAATAAAATATGAAATAAGTATGATGCACGATGATACGCTATTGAGAAAACATACT   60
      K  G  I  K  Y  E  I  S  M  M  H  D  D  T  L  L  R  K  H  T

  61  CTTAAAATTGGATCTACTTATATATTCGATCGCCATGGACATAGTAATACATATTATTCA  120
      L  K  I  G  S  T  Y  I  F  D  R  H  G  H  S  N  T  Y  Y  S

 121  AAATATGATTTTTAAAAATTTAAAATATATTATCCCTTCAGTGACCGTAGTCAAATAACA  180
      K  Y  D  F  •

 181  AACAACACCATGAGAGCTATAATTCTCACAGTTTTGTTCATTAATAGTATAAATGCTACA  240
               M  R  A  I  I  L  T  V  L  F  I  N  S  I  N  A  T

 241  ATAACTAGTTATAAGTTTGAATCCGTCAATTTTGATTCCAAAATTGAATGGACTGGGAAT  300
      I  T  S  Y  K  F  E  S  V  N  F  D  S  K  I  E  W  T  G  N

 301  GGTCTATACAATATATCCCTTAGAAATTATGGAATAAAGACGTGGCAAACGATGTATACA  360
      G  L  Y  N  I  S  L  R  N  Y  G  I  K  T  W  Q  T  M  Y  T

 361  AATGTACCAGAGGGAACATACGACATATCCGGATTTCCAAATAATGATTTCGTATCTTTC  420
      N  V  P  E  G  T  Y  D  I  S  G  F  P  N  N  D  F  V  S  F

 421  TGGGTTAAATTTGAACAAGGCGATTATAAAGTGGACAAGTATTGTACGGGACTATGCATC  480
      W  V  K  F  E  Q  G  D  Y  K  V  D  K  Y  C  T  G  L  C  I

 481  GAAGTAAAAATTGGACCACCGACTGTAACATTGACTGAATACGACGACCATATCAATTTG  540
      E  V  K  I  G  P  P  T  V  T  L  T  E  Y  D  D  H  I  N  L

 541  TACATCGAGCATCCGTATGCCACTAGAGGTAGCAAGAAGATTCCTATTTATAAACGCAAT  600
      Y  I  E  H  P  Y  A  T  R  G  S  K  K  I  P  I  Y  K  R  N

 601  GACATGTGTGATATCTACTTGTTGTATACGGCTAACTTCACATTCGGAGACTCTGAAGAA  660
      D  M  C  D  I  Y  L  L  Y  T  A  N  F  T  F  G  D  S  E  E

 661  CCAGTAATATATGATATTGATGACTACGATTGTACGTCTACAGGTTGCAGTATAGACTTT  720
      P  V  I  Y  D  I  D  D  Y  D  C  T  S  T  G  C  S  I  D  F

 721  GCCACAACAGAAAAAGTGTGTGTTATGGCACAGGGAGCCACAGAAGGGCTTCTTGATAAA  780
      A  T  T  E  K  V  C  V  M  A  Q  G  A  T  E  G  L  L  D  K

 781  ATTACGCCATGGAGTTCGGAAGTATGTCTGACACCTAAAAAGAATGTATATACATGCGCA  840
      I  T  P  W  S  S  E  V  C  L  T  P  K  K  N  V  Y  T  C  A

 841  ATTAGATCCAAAGAAGATGTTCCCAATTTCAAGGAAAAAATGACCAGAGTTATCAAGAGA  900
      I  R  S  K  E  D  V  P  N  F  K  E  K  M  T  R  V  I  K  R

 901  AAATTTAATAAACAGTCACACTCGTATTTGACCAAATTTCTCGGTAGTACATCAAATGAT  960
      K  F  N  K  Q  S  H  S  Y  L  T  K  F  L  G  S  T  S  N  D

 961  ATTACAACTTTTCTTAGCATGCTTGACTAAATATTCATAATGAACTCTCTAAACTATTTT 1020
      I  T  T  F  L  S  M  L  D  •

1021  TATCAAAAAATAAAACAATCACTAGTATTAATTTTGATAACGTAGCATAATCAACATAAC 1080

1081  CTCATCATCTATTAACTGAT  1100
```

B.

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EV   MR. AIILTVL FINSINATIT SYKFESVNFD SKIEWTGNGL YNISLRNYGI  49
VV   MRYIIILAVL FINSIHAKIT SYKFESVNFD SKIEWTGDGL YNISLKNYGI  50
VAR  MR. SVMLTVL LINSINATIT SYKFESVNFD SKIEWTGDGL YNISLKNYGI  49

EV   KTWQTMYTNV PEGTYDISGF PNNDFVSFWV KFEQGDYKVD KYCTGLCIEV  99
VV   KTWQTMYTNV PEGTYDISAF PKNDFVSFWV KFEQGDYKVE EYCTGLCVEV 100
VAR  KTWQTMYTNV PEGTYDISGF PKNDFVSFWV KFEQGDYKVE EYCTGLCIEV  99

EV   KIGPPTVTLT EYDDHINLYI EHPYATRGSK KIPIYKRNDM QDIYLLYTAN 149
VV   KIGPPTVTLT EYDDHINLYI EHPYATRGSK KIPIYKRGDM QDIYLLYTAN 150
VAR  KIGPPTVTLT EYDGHINLYI EHPYATRGSK KIPIYKRNDM QDIYLLYTAN 149

EV   FTFGDSEEPV IYDIDDYDCT STQQSIDFAT TEKVCVMAQG ATEGLLDKIT 199
VV   FTFGDSEEPV TYDIDDYDCT STQQSIDFAT TEKVCVTAQG ATEGFLEKIT 200
VAR  FTFGDSEEPV TYNIDDYDCT STQQSIDFAT TEKVCVTAQG ATEGFLEKIT 199

EV   PWSSEVQLTP KKNVYTCAIR SKEDVPNFKE KMTRVIKRKF NKQSHSYLTK 249
VV   PWSSEVQLTP KKNVYTCAIR SKEDVPNFKD KMARVIKRKF NKQSQSYLTK 250
VAR  PWSSEVQLTP KKNVFTCAIR SKEDVSNFKD KMTRVIKRKF NKQSQNYMTK 249

EV   FLGSTSNDIT TFLSMLD          266
VV   FLGSTSNDVT TFLSMLNLTK YS    272
VAR  FLGTTANDVT TVISMLD          266
```

Figure V-3. Ectromelia virus IFN-γ receptor homolog binds multiple IFN-γ species. Supernatants from ectromelia virus infected cells (lanes 1, 3 and 5) and mock infected cells (lanes 2, 4 and 6) were chemically cross-linked to [^{32}P]-rabbit, -human, and -murine IFN-γ (lanes 1-2, 3-4, 5-6, respectively). Location of monomer and dimer forms of IFN-γ are indicated with arrows, as is the labeled complex between the ectromelia virus IFN-γ binding protein and the labeled ligand. Molecular weight marker sizes are indicated on the left.

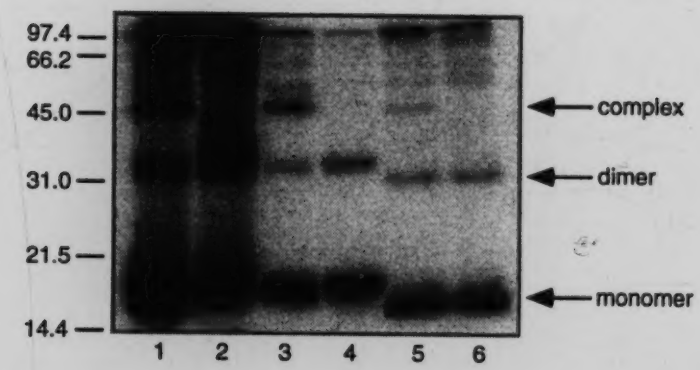

97.4
66.2
45.0
31.0
21.5
14.4
1
2
3
4
5
6
complex
dimer
monomer

Figure V-4. Species specificity of the ectromelia virus IFN-γ binding protein. Supernatants from ectromelia virus infected cells were chemically crosslinked to (A) [^{32}P] human IFN-γ, (B) [^{32}P] murine IFN-γ and (C) [^{32}P] rabbit IFN-γ. In all panels, chemical cross-linking was performed in the absence (lane 1) or presence (lanes 2-13) of increasing amounts of unlabeled IFN-γ, as illustrated. Addition of unlabeled competitor IFN-γ was in 5x, 10x, 20x, and 40x excess over the amount of labeled IFN-γ. Location of monomer and dimer forms of IFN-γ are indicated on the right, while molecular weight marker sizes are indicated on the left.

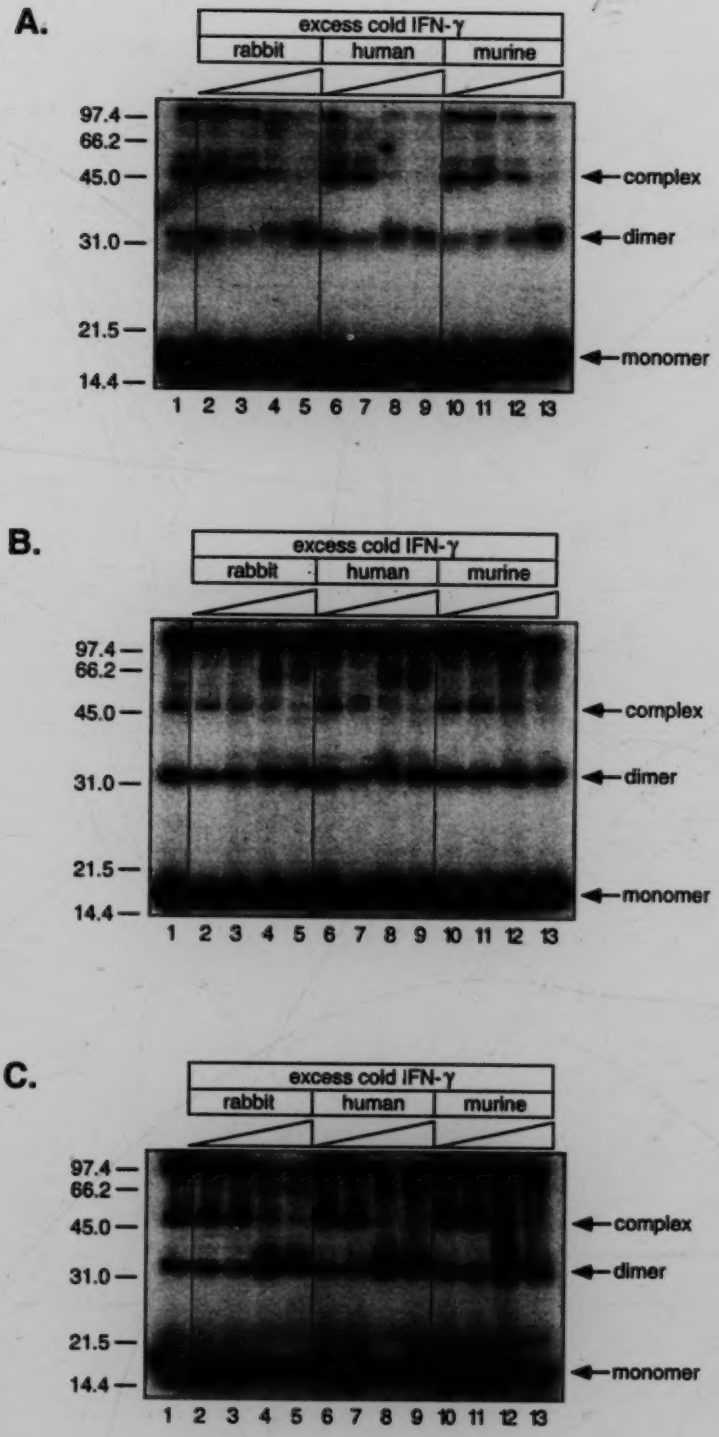
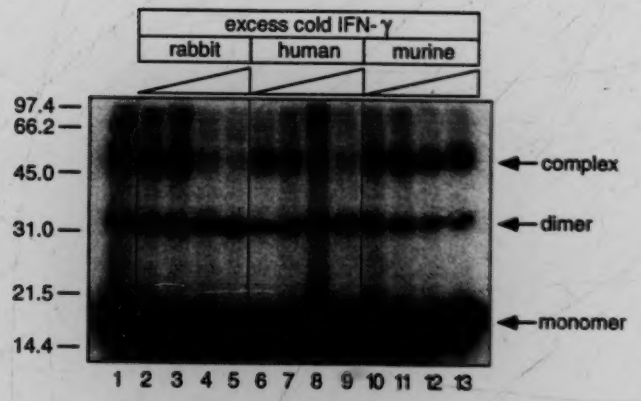
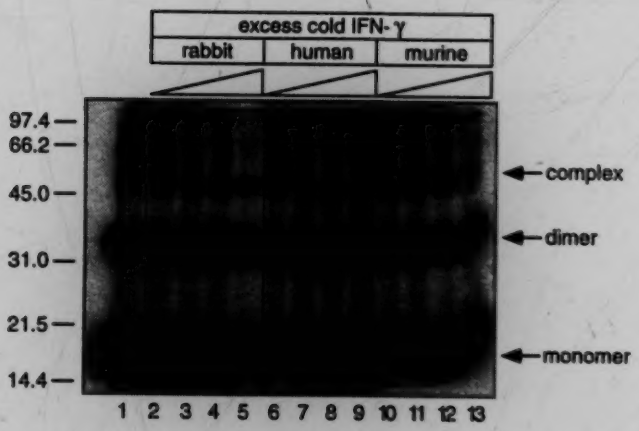

A.
excess cold IFN-γ
rabbit
human
murine
97.4
66.2
45.0
31.0
21.5
14.4
complex
dimer
monomer
1 2 3 4 5 6 7 8 9 10 11 12 13
B.
excess cold IFN-γ
rabbit
human
murine
97.4
66.2
45.0
31.0
21.5
14.4
complex
dimer
monomer
1 2 3 4 5 6 7 8 9 10 11 12 13
C.
excess cold IFN-γ
rabbit
human
murine
97.4
66.2
45.0
31.0
21.5
14.4
complex
dimer
monomer
1 2 3 4 5 6 7 8 9 10 11 12 13

Figure V-5. Species specificity of the vaccinia virus IFN-γ binding protein. Supernatants from vaccinia virus infected cells were chemically crosslinked to (A) [^{32}P] human IFN-γ, (B) [^{32}P] murine IFN-γ and (C) [^{32}P] rabbit IFN-γ. In all panels, chemical cross-linking was performed in the absence (lane 1) or presence (lanes 2-13) of increasing amounts of unlabeled IFN-γ, as illustrated. Addition of unlabeled competitor IFN-γ was in 5x, 10x, 20x, and 40x excess over the amount of labeled IFN-γ. Location of monomer and dimer forms of IFN-γ are indicated on the right, while molecular weight marker sizes are indicated on the left.

A.


excess cold IFN-γ
rabbit
human
murine
97.4
66.2
45.0
31.0
21.5
14.4
complex
dimer
monomer
1 2 3 4 5 6 7 8 9 10 11 12 13

B.


excess cold IFN-γ
rabbit
human
murine
97.4
66.2
45.0
31.0
21.5
14.4
complex
dimer
monomer
1 2 3 4 5 6 7 8 9 10 11 12 13

C.

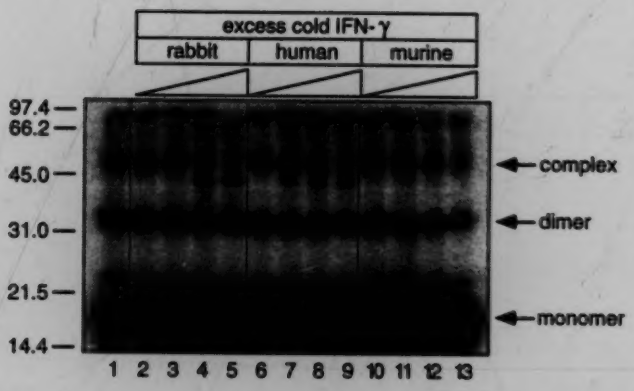
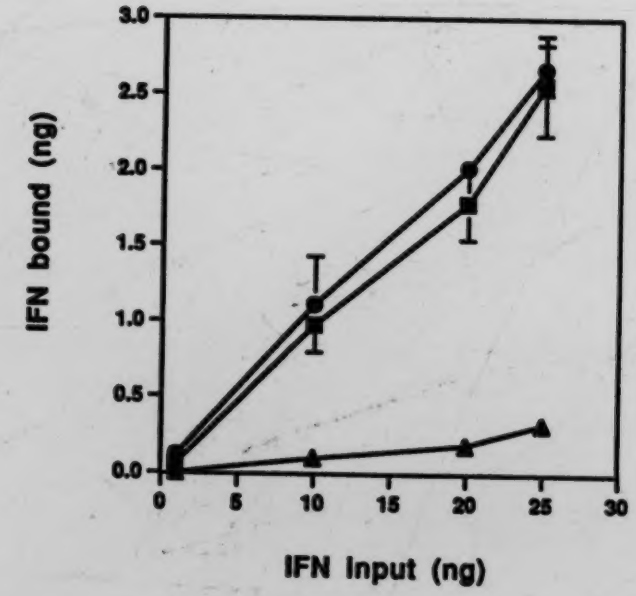

excess cold IFN-γ
rabbit
human
murine
97.4
66.2
45.0
31.0
21.5
14.4
complex
dimer
monomer
1 2 3 4 5 6 7 8 9 10 11 12 13

Figure V6. IFN-γ binding specificity of primate BGMK cells. Binding analysis of [^{32}P] human IFN-γ (■), [^{32}P] rabbit IFN-γ (●), and [^{32}P] murine IFN-γ (▲), to the primate IFN-γ receptor on intact BGMK cells. Specific binding reflects total binding of radiolabeled ligand minus non-specific binding in the presence of 100-fold excess unlabeled ligand. All binding assays were done in triplicate.


IFN bound (ng)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
25
30
IFN Input (ng)

References

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CHAPTER VI

Disruption of M-T5, a Novel Myxoma Virus Gene Member of the Poxvirus Host Range Superfamily, Results in Dramatic Attenuation of Myxomatosis in Infected European Rabbits[1]

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Introduction

Survival of a virus within a susceptible host requires specific strategies to evade antiviral and inflammatory immune responses elicited against a viral infection (2, 13, 28, 31, 39). Poxviruses are among the largest of eukaryotic DNA viruses (32), and have proven to be an excellent system in which to study immune evasion for several reasons. Poxviruses encode more proteins than are necessary for progeny virion formation, and many of these extra non-essential proteins are used primarily for subverting host immune defences within the vertebrate host. Poxviral immune evasion strategies include interference with cytokines and growth factors, inhibition of the complement cascade, reduction of inflammation, and repression of cellular immune recognition (2, 4, 13, 28, 31, 39, 41, 45).

The substantial encoding capacity of the poxviral genome allows for adaptation to multiple cellular environments, which may explain the broad host range characteristic of many poxviruses. Vaccinia virus, an orthopoxvirus whose evolutionary history remains obscure, is capable of replicating in a broad range of mammalian and avian cells, but is defective for replication in Chinese hamster ovary (CHO) cells (9). Cowpox virus, a related orthopoxvirus which is capable of replicating in CHO cells, encodes a 77 kDa protein, CHOhr, which can confer to recombinant vaccinia virus constructs the ability to productively infect CHO cells (40). In addition to the cowpox virus CHOhr gene, two additional related but distinct vaccinia virus genes, C7L and K1L, have been implicated in the determination of vaccinia virus host range *in vitro* (36). Ectromelia virus and rabbitpox virus also encode related proteins involved in host range restriction (1, 5), indicating the existence of a larger superfamily of viral genes dedicated to the control of poxvirus host range. While proteins encoded by this family of poxvirus genes have been defined by virtue of their

ability to determine host range in cultured cells, their relevance *in vivo* is speculative, since the natural host(s) of many poxviruses remain unknown.

Myxoma virus is one of the few poxviruses whose natural history has been well established, and thus provides an excellent model system in which to study virus-host interactions. Myxoma virus expresses a diverse range of virulence factors, which interact with elements of the host immune system in the establishment of a myxomatosis disease state (30). Some of these virulence factors subvert immune regulation by modulating host cytokine networks (refer to Introduction). Here we describe the identification and characterization of a novel myxoma virus gene, M-T5, which encodes a protein that contains significant homology with members of the poxviral family of host range proteins. Deletion analysis of the M-T5 open reading frame indicates that one of the functions of the M-T5 protein is to allow the productive infection of rabbit peripheral lymphocytes, and in the absence of M-T5 expression, such cells undergo a rapid apoptotic response. Thus, the myxoma virus M-T5 protein functions as a critical virulence factor in the establishment of myxomatosis in the European rabbit by permitting virus spread via infected lymphocytes and thus dissemination to multiple internal and external peripheral locations.

Results

Myxoma M-T5 Possesses Homology to Poxviral Host Range Proteins.

Due to the overall high homology between members of the *Leporipoxvirus* genus, sequencing of the myxoma virus M-T5 open reading frame from genomic DNA was facilitated by utilizing primers initially directed towards the published Shope fibroma virus S-T5 sequences (46, 48). The myxoma M-T5 open reading frame is present as two copies, one within each virus terminal inverted repeat, and consists of 1452 nucleotides which encodes a protein of 483 amino acids (Figure VI-1). M-T5, which has a predicted molecular weight of 55 kDa, possesses neither a signal peptide sequence nor sites for N-linked glycosylation, but does contain a single ankyrin-like repeat (Figure VI-1). Ankyrins bind integral membrane proteins, presumably through ankyrin repeat domains (21, 22), but the significance of the single M-T5 ankyrin-like repeat has yet to be determined.

With the exception of the ankyrin-like repeat, database searching revealed no significant homology between the myxoma virus M-T5 protein and any non-viral proteins. However, a low but significant level of homology was observed between M-T5 and a variety of poxviral proteins, many of which have been defined by their ability to mediate host range (Table VI-1). Cowpox virus, in contrast to vaccinia virus, is capable of multiplying in CHO cells, and the expression of the cowpox virus CHOhr transgene product is sufficient to allow vaccinia virus replication in CHO cells (9, 40). Vaccinia virus encodes two additional open reading frames, C7L and K1L, which express proteins required for productive replication in human cells, and whose deletion can be compensated for by expression of the CHOhr gene product (36). While in some

cell lines the C7L and K1L gene products appear to possess overlapping functions, in other cell lines only one gene product is critical for viral replication (33, 42). An ectromelia virus homolog of the vaccinia virus K1L gene, encoded by the ectromelia virus K1L open reading frame, was found to permit viral replication in several cell lines, but have no effect on *in vivo* replication and spread in infected mice (5). In addition, rabbitpox virus encodes three serine protease inhibitor homologs, one of which, SPI-1, has been implicated in both the determination of host range and the formation of hemorrhagic lesions (1).

The myxoma virus M-T5 protein contains the highest degree of sequence similarity with the vaccinia virus B4R gene product, and its variola virus homolog, the B6R gene product (Table VI-1) (12, 25). While little is known concerning the function of the vaccinia virus B4R gene product, homology between this protein and the cowpox virus CHOhr encoded protein has been noted (16). Figure VI-2 illustrates an alignment between the myxoma virus and Shope fibroma virus T5 proteins, the vaccinia virus B4R gene product and the cowpox virus CHOhr gene product. It should be noted that while the homology between these sequences extends across the entire length of the proteins, both the myxoma virus and Shope fibroma virus T5 proteins contain a substantial deletion within the central region, as compared with the other poxviral proteins. The significance of this apparent deletion remains to be determined.

Construction and Characterization of M-T5$^-$ Recombinant Myxoma Viruses.

Due to the location of the M-T5 open reading frame within the terminal inverted repeats of the myxoma virus genome, it is present as two copies, and maps within the Bam K restriction fragment (Figure VI-3a). In order to inactivate both copies of the M-T5 gene, a dominant selection method involving the *E. coli*

guanine phosphoribosyltransferase (Ecogpt) was used. This selection method was shown to be useful in the construction of poxviral recombinants (3, 10), including those of myxoma virus (24, 47 & Chapter IV). Disruption of the M-T5 open reading frame by PCR amplification and subsequent insertion of a cassette containing the Ecogpt gene under the control of the vaccinia virus p7.5 promoter is outlined (Figure VI-3b). Recombinant M-T5gpt was transfected into both wild-type (strain Lausanne) myxoma virus and vMyxlac infected cells, in order to create two M-T5$^-$ mutants, vMyxT5$^-$ and vMyxlacT5$^-$, respectively, in both the wild-type and vMyxlac backgrounds. Due to the presence of the *E. coli* lacZ gene within the genome of vMyxlac, visualization of viral foci with this background is greatly facilitated by X-Gal staining. We have recently determined that the overall virulence of vMyxlac is slightly less than that ascribed to the original Lausanne isolate of myxoma (Chapter IV), and thus the parental strain of myxoma virus used to create recombinant viruses may influence virulence studies *in vivo*. As a control, therefore, an M-T5 revertant virus was constructed in which the intact M-T5 open reading frame was reconstructed back into the M-T5$^-$ mutant virus, and is refered to as vMyxT5rev (Chapter II).

To ensure disruption of both copies of the M-T5 gene in vMyxT5$^-$ and vMyxlacT5$^-$, and complete restoration in vMyxT5rev, PCR analysis was performed on DNA isolated from plaque purified virus. As shown in Figure VI-3b, wild-type M-T5 and mutant M-T5gpt genes possess identical sequences at their terminal ends, to which the M-T5 5' and S-T5 3' primers hybridize. Wild-type M-T5 (1452 bp) is easily distinguished from mutant M-T5gpt (1590 bp) based on size by agarose gel electrophoresis (Figure VI-4, lanes 2 and 3, respectively). DNA derived from plaque purified vMyxT5$^-$ and vMyxlacT5$^-$ demonstrated only the disrupted M-T5gpt sequence (Figure VI-4, lanes 4 and 5,

respectively), while DNA derived from plaque purified vMyxT5rev demonstrated only the wild-type M-T5 sequence (Figure VI-4, lane 6).

Defective Growth of M-T5$^-$ Virus in a CD4$^+$ Rabbit T-cell Line (RL5).

No defects were noted in the ability of vMyxlacT5$^-$ to replicate in cultured rabbit RK13 fibroblasts *in vitro* in a single step growth curve analysis (Figure VI-5a). Similar results were also found in cultured primate BGMK cells (data not shown). However, vMyxlacT5$^-$ was found to be defective for replication in a cultured rabbit RL5 CD4$^+$ T-cell line (Figure VI-5b). Initial studies concerning the host range restriction of vaccinia virus in CHO cells suggested that early and extensive inhibition of host and viral protein synthesis may be responsible for the abortive infection (9, 17). Due to the sequence similarities observed between myxoma virus M-T5 and known poxviral host range proteins, protein synthesis was analyzed following vMyxlacT5$^-$ infection in replication permissive RK13 fibroblasts and non-permissive RL5 lymphocytes. No significant differences were noted in host protein shutoff or viral protein synthesis between vMyxlac and vMyxlacT5$^-$ infected RK13 cells at early or late times of infection (Figure VI-6a). However, both host and viral protein synthesis appeared to be severely inhibited early after vMyxlacT5$^-$ infection of RL5 cells (Figure VI-6b). Significant protein synthesis inhibition was observed as early as one hour post-infection (Figure VI-6b, compare lanes 2 and 3), and appeared virtually complete by four hours post-infection (Figure VI-6b, compare lanes 6 and 7). In both RK13 and RL5 cells, infection with the control virus vMyxT5rev was indistinguishable from that of vMyxlac (Figure VI-6a and b, lane 10). Similar results were found in both cell lines with vMyxT5$^-$, indicating that *in vitro*, the two M-T5$^-$ mutant viruses were indistinguishable (data not shown). Thus, it appears that in RL5 lymphocytes, inhibition of both host and viral protein synthesis

directly correlates with the abortive infection observed in the growth curve analysis.

In order to determine if the extensive early shutoff of host and viral protein synthesis in RL5 cells is unique to the myxoma virus M-T5$^-$ mutant, metabolic labeling of RL5 cells infected with some of the other characterized myxoma virus mutants was performed (Figure VI-6c). Mutants of M-T2, a secreted homolog of the mammalian tumor necrosis factor receptor (47), M11L, a receptor-like molecule with no known cellular homolog (14, 34), M-T7, a secreted homolog of the mammalian interferon-γ receptor (ref. 49 & Chapter VI) and SERP-1, a serine proteinase inhibitor (24), were compared to vMyxlac and vMyxlacT5$^-$ at early (Figure VI-6c, lanes 2-7) and late (Figure VI-6c, lanes 9-14) times of infection. vMyxlacT5$^-$ appeared to be the only characterized myxoma virus mutant to cause inhibition of both host and viral protein synthesis in infected RL5 lymphocytes. This result was of relevance since both the T2$^-$ and M11L$^-$ mutant myxoma viruses are known to be defective for replication in RL5 cells (23).

Rabbit Lymphocytes Undergo Apoptosis in Response to Infection by M-T5$^-$ Mutant Myxoma Viruses.

Apoptosis, or programmed cell death, is known to play a physiologically important role in such diverse processes as embryonic development, tumorigenesis and lysis of virally infected cells (20, 50). Recently, vaccinia virus infection of CHO cells was shown to induce apoptosis (18). Due to the similarities between infection of CHO cells with vaccinia virus and infection of RL5 cells with myxoma T5$^-$ mutant virus, we initiated studies to see if vMyxlacT5$^-$ infection of RL5 cells would lead to apoptosis. A characteristic feature of apoptosis is the degradation of DNA to oligonucleosomal fragments

(52), which may be observed as DNA laddering when cell lysates are subjected to agarose gel electrophoresis. DNA laddering was observed in lysates from RL5 cells infected with vMyxlacT5$^-$, but not in lysates from RL5 cells either mock infected, or infected with vMyxlac or vMyxT5rev (Figure VI-7). As with the shutoff of host and viral protein synthesis, DNA fragmentation was observed as early as two hours following vMyxlacT5$^-$ infection (Figure VI-7, lane 3). No fragmentation was observed at any time following infection of RK13 cells with these same viruses in a parallel study (data not shown).

DNA fragmentation was also measured in RL5 cells by quantitating the terminal deoxynucleotidyl transferase-mediated incorporation of fluorescein-12-dUTP into DNA using flow cytometry analysis. Flow cytometry analysis of a timecourse of vMyxT5$^-$ infected RL5 cells demonstrated that by 4, 8 and 12 hours post-infection 20%, 41% and 50% of cells, respectively, exhibited enhanced fluorescence caused by DNA fragmentation (Table VI-2). In contrast, at 12 hours post-infection, mock infected RL5 cells and vMyx infected RL5 cells exhibited 1% and 10% enhanced fluorescence, respectively (Table VI-2). Infection of RL5 cells with vMyxT5rev resembled that of RL5 cells infected with vMyx (data not shown). vMyxT5$^-$ infection of RL5 cells beyond 12 hours resulted in a dramatic reduction of the number of harvested cells, such that flow cytometric analysis became difficult to perform. We conclude that abortive infection of the myxoma M-T5$^-$ mutant virus in RL5 lymphocytes results in the apoptotic death of these cells.

Infection of Primary Rabbit Lymphocytes and Monocytes with Myxoma M-T5$^-$ Virus.

While the amount of apoptosis seen in RL5 cells infected with vMyxlacT5$^-$ is striking, RL5 is a T-lymphoma cell line derived from a herpesvirus ateles-induced rabbit tumor (19). Thus, to ascertain whether or not apoptosis is a feature of vMyxlacT5$^-$ infected primary rabbit leukocytes *in vivo*, peripheral blood mononuclear leukocytes were harvested, fractionated into adherent and non-adherent cells, and subjected to flow cytometry analysis following infection with the various myxoma virus recombinants (Figure VI-8). In peripheral lymphocytes infected with vMyxlacT5$^-$, a distinct population of cells demonstrated an increase in relative fluorescence due to DNA fragmentation, which routinely corresponded to 20-30% apoptosis (Figure VI-8a). The scatter profile of vMyxlacT5$^-$ infected lymphocytes also differed from that of mock, vMyxlac and vMyxT5rev infected lymphocytes, consistent with the appearance of a population of smaller, apoptotic cells. Similar to the case of RL5 cells, primary lymphocytes infected with vMyxlacT5$^-$ also routinely exhibited a decrease in total number of cells harvested. In contrast, adherent cells, enriched for circulating monocytes, showed no significant increase in relative fluorescence following infection with vMyxlac, vMyxlacT5$^-$ or vMyxT5rev, as compared to mock infected cells (Figure VI-8b). In all of these experiments, no differences were found in the levels of DNA fragmentation when wild-type myxoma and vMyxT5$^-$ were used in the place of vMyxlac and vMyxlacT5$^-$, respectively (data not shown). Thus we conclude that infection of primary rabbit peripheral lymphocytes, but not cells of the monocyte/macrophage lineage, with a myxoma M-T5$^-$ mutant virus results in apoptosis and abortive infection.

Myxoma Virus M-T5 Is a Potent Virulence Factor in European Rabbits.

Myxoma virus infection of European rabbits induces a rapidly progressive and lethal disease known as myxomatosis (11). Infection of European rabbits with wild-type myxoma virus (strain Lausanne) is routinely 100% fatal, whereas infection of European rabbits with the vMyxlac derivative strain expressing the lacZ cassette results in approximately 95% mortality with a slight attenuation of disease symptoms (Chapter IV). Since no significant differences were observed between *in vitro* infections with vMyxT5$^-$ (wild-type background) and vMyxlacT5$^-$ (vMyxlac background), vMyxT5$^-$ was chosen for comparison with wild-type myxoma virus to determine the effect of an M-T5 disruption in myxoma virus virulence *in vivo*.

As outlined in Table VI-3, a dramatic reduction in virulence was observed in rabbits infected with vMyxT5$^-$ as compared to rabbits infected with wild-type myxoma virus. At day 4 post-inoculation, primary site lesions in rabbits infected with vMyxT5$^-$ were consistently smaller and firmer than those in rabbits infected with wild-type myxoma virus. By day 7 post-inoculation, by which time rabbits infected with wild-type myxoma virus demonstrated all of the classical symptoms of myxomatosis, including Gram negative bacterial infection of nasal and conjunctival mucosa, and multiple secondary skin lesions, rabbits infected with vMyxT5$^-$ remained essentially asymptomatic, with no evidence of viral spread to secondary sites. By day 10 post-inoculation, all four rabbits infected with wild-type myxoma virus had to be euthanized (2 rabbits each on days 9 and 10), while all rabbits infected with vMyxT5$^-$ demonstrated no overt signs of myxomatosis, save for one minute secondary lesion on the ear of one rabbit. The primary lesions of vMyxT5$^-$ infected rabbits were fully resolved by 14 days post-inoculation, and these rabbits were immune to subsequent challenge by wild-type myxoma virus. Rabbits infected with the control virus, vMyxT5rev, in which the T5gpt disruption was reconstituted back to wild-type, displayed a

disease state indistinguishable from rabbits infected with wild-type myxoma virus (data not shown). Thus, we conclude that M-T5 is a potent virulence factor which plays a critical role in the pathogenesis of myxoma virus in European rabbits, and in the M-T5$^-$ deletion mutant, the virus spreads poorly, if at all, to secondary sites.

Histological Analysis of Rabbits Infected with Wild-type and M-T5$^-$ Mutant Myxoma Viruses.

Due to the significant difference in the pathogenic profile of the M-T5$^-$ mutant virus versus the M-T5$^+$ parent or M-T5rev myxoma viruses, a more detailed histological analysis was performed on tissue sections from vMyxlac and vMyxlacT5$^-$ mutant virus infected rabbits harvested at days 4, 7 and 10 post-inoculation (summarized in Table VI-4). The most striking difference between wild-type myxoma virus and M-T5$^-$ mutant virus infection was observed at the primary site of inoculation (Figure VI-9). At day 4 post-inoculation, primary lesions from rabbits infected with vMyxlac (Figure VI-9a) demonstrated considerable edema with a minor accumulation of heterophils. In contrast, while primary lesions from rabbits infected with vMyxlacT5$^-$ (Figure VI-9b) displayed little edema, a significant infiltration of heterophils was observed throughout the dermis. Heterophils function as the primary line of cellular defence early within an inflammatory reaction, and are subsequently replaced with mononuclear lymphocytes as the inflammatory reaction progresses. Also, while parental vMyxlac infected primary lesions showed a slight disorganization of the epithelium, vMyxlacT5$^-$ infected primary lesions demonstrated a zone of actively mitotic basal keratinocytes within the inner epithelium, which was effectively replacing degenerating cells of the outer epithelium with a new epithelial layer.

At day 7 post-inoculation, primary lesions from rabbits infected with parental vMyxlac demonstrated considerable edema and hemorrhage within the dermis, necrosis and degranulation of accumulating heterophils, and a necrotic epithelium which was beginning to lift off the basement membrane (Figure VI-9c). Primary lesions from rabbits infected with vMyxlacT5$^-$, however, demonstrated a normal epidermal surface, replacement of heterophils with mononuclear lymphocytes, and neither edema nor hemorrhage within the dermis (Figure VI-9d). By day 10 post-inoculation, when vMyxlac infected primary lesions continued to display extensive edema, hemorrhage and necrosis of heterophils, along with a completely disorganized epithelial layer (Figure VI-9e), the site of inoculation with vMyxlacT5$^-$ (Figure VI-9f) essentially resembled a parallel section harvested from an uninfected control rabbit (data not shown). Thus, it appears that within primary lesions of rabbits infected with parental M-T5$^+$ myxoma virus, an inflammatory reaction capable of controlling viral infection is not established, whereas in primary lesions of rabbits infected with the M-T5$^-$ mutant myxoma virus, a very rapid and effective inflammatory reaction results in complete resolution of the infection by day 10 post-inoculation, with no development of secondary lesions.

Since complete resolution of M-T5$^-$ mutant myxoma virus infection appeared to occur by 7 to 10 days at the primary site of inoculation, it is useful to consider whether the lymph node and spleen may have played a critical role in the resolution process. Infection of rabbits with vMyxlac lead to mild reactivity within the lymph nodes and spleen by day 4 post-inoculation (Table VI-4), as immature lymphocytes replaced mature lymphocytes that began to migrate out of the lymphoid organs. By day 7 post-inoculation, very few mature lymphocytes were found within the lymphoid organs of rabbits infected with vMyxlac, and it appeared that the replication of stem cells was insufficient to

replace the mature lymphocytes, resulting in the net outward migration of immature cells. At day 10 post-inoculation, even fewer lymphocytes were found within the germinal centers of the lymphoid organs of vMyxlac infected rabbits, and within the spleen, a significant level of lymphocyte necrosis was observed, perhaps corresponding to productive replication of virus in this organ. In stark contrast, at days 4 and 7 post-inoculation, no cellular reactivity was observed within the lymph nodes and spleen of rabbits infected with vMyxlacT5$^-$. At day 10 post-inoculation, however, mild reactivity characterized by increased mitotic activity within the germinal centers of both lymphoid organs was observed, along with the appearance of macrophages and a high level of mature lymphocytes. Given the histological and pathogenic profile of the M-T5$^-$ mutant myxoma virus at day 10 post-inoculation, this reactivity is consistent with the recognition and formation of memory T lymphocytes in response to viral antigen. Thus, the early resolution of the M-T5$^-$ mutant myxoma virus within the primary site of infection appeared not to be accompanied by the development of antigen-activated lymphocytes within the secondary immune organs.

Consistent with the absence of gross lesions at peripheral locations in M-T5$^-$ mutant myxoma virus infected rabbits, and the rapid resolution of infection at the primary site of inoculation, no lesions were found upon histological analysis of tissues harvested from outside the primary inoculation site in vMyxlacT5$^-$ infected rabbits (Table VI-4). In contrast, by day 7 post-inoculation rabbits infected with vMyxlac demonstrated extensive secondary lesions, established by virtue of the dissemination of infectious virus within productively infected lymphocytes. Thus, we conclude that wild-type myxoma virus is capable of disseminating via infected lymphocytes to peripheral areas and establishing secondary sites of infection, whereas M-T5$^-$ mutant myxoma virus is incapable of disseminating from the primary inoculation site because a rapid supervening

apoptotic response in infected lymphocytes prevents the transit of infectious virus through the lymphoreticular system to secondary sites.

Discussion

In order to efficiently replicate and establish a productive infection, myxoma virus produces a wide range of immunomodulatory proteins which perform diverse functions (29, 30). In this study, we report on the role of the myxoma virus M-T5 protein, which along with the S-T5 protein from the antigenically related SFV (46), contains significant homology to a larger family of poxviral proteins implicated in the determination of host range (1, 5, 36, 40). Interestingly, both the myxoma virus and SFV T5 proteins have internal deletions of 106 amino acids when compared to the cowpox virus CHOhr encoded protein, the most extensively studied of the poxviral host range proteins. The myxoma virus M-T5$^-$ mutant failed to replicate on cultured rabbit T lymphocytes while displaying wild-type replication on cultured rabbit kidney fibroblasts, and it is likely that one of the roles of M-T5 is to extend the tissue range of myxoma virus to cells of lymphoid origin. Myxoma virus displays a very restricted "host" range in nature, as only members of the family *Leporidae* are infected by myxoma virus, and only members of the genus *Oryctolagus* are susceptible to myxomatosis (11). As such, the myxoma virus M-T5 protein defines cell restriction within its natural host, as opposed to the classic definition of host range meaning the ability to infect a heterologous species. Thus, a more accurate description would be that M-T5 imparts tissue or cell type specificity, rather than host range.

An important factor in the extreme virulence of myxomatosis is the ability of myxoma virus to replicate efficiently in lymphoid cells, allowing for dissemination of the virus and establishment of internal and external secondary sites of infection. The myxoma virus M-T5 protein appears to be involved in allowing productive replication of myxoma virus in rabbit lymphocytes. In cultured rabbit CD4$^+$ T-cells (RL5 cells) or primary lymphocytes, infection with

an M-T5$^-$ mutant virus results in rapid and extensive programmed cell death, or apoptosis (20), coupled with a rapid inhibition of both host and viral protein synthesis. Since viral infection is capable of inducing apoptosis in certain circumstances (20, 50), many viruses, including adenoviruses, herpesviruses, baculoviruses, and poxviruses are known to produce specific viral gene products responsible for blocking the apoptotic response (7, 8, 15, 43, 51). While some viral proteins, such as the cowpox virus CrmA protein, inhibit cell death by directly blocking specific apoptotic pathways (43), the myxoma virus M-T5 protein appears instead to ensure that lymphocytes do not undergo an abortive infection, due to a non-specific shutoff of protein synthesis, which is likely the stimulus that leads to programmed cell death. Infection of non-permissive CHO cells with vaccinia virus, insect SF-21 cells with baculovirus, or SK-N-SH neuronal cells with certain herpes simplex 1 virus mutants, also result in the global inhibition of all protein synthesis and subsequent apoptotic cell death (7, 8, 43). In the case of the herpes simplex 1 $\gamma_1$34.5$^-$ mutant, it has been shown that the protein kinase PKR complexes with a 90 kDa phosphoprotein, leading to overall protein synthesis shut-off via phosphorylation of eIF-2α (6).

The myxoma virus M-T5 protein is not unique in influencing viral replication in rabbit lymphocytes, as at least two additional myxoma virus proteins appear to play a role in allowing a fully productive infection. Infection of RL5 lymphocytes with mutants of either the M-T2 protein, a soluble homolog of the mammalian tumor necrosis factor receptor (47), or the M11L protein, a membrane-associated protein of unknown function (14, 34), also results in the inhibition of viral replication and subsequent death of infected cells via apoptosis (23). However, a critical difference is observed upon infection of lymphocytes with these three myxoma virus mutants: the M-T5$^-$ mutant virus is the only mutant which results in the global inhibition of both host and viral

protein synthesis. As demonstrated by flow cytometry analysis, infection of primary peripheral blood leukocytes with the M-T5$^-$ mutant virus resulted in the apoptotic death of a population of lymphocytes, while causing no detectable apoptosis in infected monocytes. Thus, it is reasonable to propose that, unlike M-T2 and M11L, the M-T5 protein ensures the continued operation of the translational machinery specifically in infected lymphocytes, and that the failure to maintain translation is itself an apoptosis inducer in these cells. Indeed, we have observed that cyclohexamide treatment alone in uninfected RL5 lymphocytes induces apoptosis (data not shown).

While the rapid inhibition of host and viral protein synthesis and subsequent programmed cell death observed upon infection of rabbit lymphocytes with an M-T5$^-$ mutant virus suggests a mechanism of M-T5 function, the biochemical properties of this protein have yet to be determined. However, an interesting correlation exists between M-T5 and the cowpox virus CHOhr, vaccinia virus K1L and ectromelia virus K1L proteins implicated in poxvirus host range determination. Infection of rabbit RL5 cells with an M-T5$^-$ mutant virus, rabbit kidney RK13 cells with K1L$^-$ mutants of vaccinia virus and ectromelia virus, and CHO cells with vaccinia virus lacking the addition of the cowpox CHOhr protein, all induce the rapid shut-off of both host and viral protein synthesis. Notably, each of these poxviral proteins contains at least one ankyrin-like repeat. Ankyrins constitute a family of proteins believed responsible in coordinating the interactions between integral membrane proteins and cytoskeletal elements, and a variety of cellular proteins involved in protein-protein interactions have been shown to contain ankyrin-like repeats (22). A number of orthopoxvirus proteins contain ankyrin-like repeats, and it has been suggested that these proteins may interact with either cellular cytoskeletal proteins, or host or viral membranes, in the formation of structures

necessary for protein synthesis and assembly of virions, thus regulating the virus growth cycle (16, 38). It will be relevant to determine whether the ankyrin-like repeat is important in the localization of the myxoma virus M-T5 protein in rabbit lymphocytes.

Myxoma virus utilizes a diverse range of proteins in order to circumvent the host immune system in the establishment of a lethal myxomatosis infection (29, 30). To date, deletion analyses of M-T2, M11L, M-T7, SERP1 and MGF have shown that these myxoma virus proteins function as virulence factors which influence the pathogenicity of myxoma virus within the European rabbit (ref. 24, 34, 35, 47 & Chapter IV). Like M-T2 and M-T7, the M-T5 open reading frame is located within the terminal region of the myxoma virus genome, where genes nonessential for viral replication in tissue culture but critical for pathogenesis and host-virus interactions *in vivo*, tend to cluster (44). While M-T5 is the third viral gene shown to be necessary for the productive infection of rabbit lymphocytes (along with M-T2 and M11L), an event which is believed to be critical for the dissemination of myxoma virus within a susceptible host, it is the first viral gene shown to be required for protein synthesis in infected lymphocytes.

Infection of European rabbits with the M-T5$^-$ mutant virus demonstrated that M-T5 is one of the most potent virulence factors yet characterized. While the mortality rate of rabbits infected with wild-type virus was 100%, all of the rabbits infected with the M-T5$^-$ mutant virus displayed virtually none of the disease symptoms of myxomatosis and fully recovered from the infection. To date, the only other myxoma mutant virus which demonstrated a recovery rate of 100% is the M11L$^-$ mutant; the other virulence factor mutant viruses demonstrated intermediate recovery rates ranging from 60% with the SERP1$^-$ mutant to >90% with the M-T7$^-$ mutant (ref. 24, 34, 47 & Chapter IV). Of

importance is the observation that rabbits infected with the M-T5$^-$ mutant virus remained essentially asymptomatic, demonstrating neither lesions at secondary sites nor establishment of a Gram negative bacterial infection, two of the hallmarks of a myxomatosis infection. Rabbits infected with any of the other characterized myxoma virus mutants, regardless of the recovery rate, demonstrated mild to severe symptoms of myxomatosis. It should also be noted that the M-T5 protein is currently the only intracellular, non-membrane associated protein which functions as a virulence factor in myxoma virus pathogenesis. Aside from the M11L protein, which is membrane associated, all other characterized virulence factors are secreted proteins.

The dramatic attenuation of the M-T5$^-$ mutant virus appears to reflect an increased ability of the host to rapidly control the infection at the primary site of inoculation. During an effective inflammatory reaction to tissue damage, heterophils function as the main early line of cellular defense, and are progressively replaced by infiltrating mononuclear cells. Heterophils in the rabbit are functionally analogous to neutrophils in humans and possess the properties of chemotaxis, adherence to immune complexes, and phagocytosis, whereas the functions of mononuclear cells include phagocytosis, killing of ingested microorganisms, antigen presentation, and secretion of enzymes, complement components and cytokines. Within the primary lesions of rabbits infected with the M-T5$^-$ mutant virus, the early accumulation of heterophils was rapidly replaced by the infiltration of mononuclear cells, suggesting the activity of a well controlled and effective inflammatory reaction to the virus infection. In fact, by day 10 post-inoculation, it was difficult to differentiate between the primary site of inoculation with the M-T5$^-$ mutant virus and a parallel section of skin tissue harvested from an uninfected control rabbit. Furthermore, this inflammatory response at the site of inoculation against the M-T5$^-$ mutant virus

was not accompanied by noticeable increases in activation of lymphocytes in either the lymph nodes or spleen, suggesting that activation of acquired cellular immunity was not a major component of virus clearance. Only at day 10 post-inoculation, when infection with the M-T5$^-$ mutant virus had been effectively controlled, was mild activation of lymphocytes in the lymphoid organs detected. This observation differs substantially from that reported for the M-T7$^-$ myxoma virus mutant, which is disrupted for the soluble interferon-γ receptor homolog, which demonstrated extensive activation of lymphocytes within the spleen and regional lymph nodes of infected animals by day 4 post-inoculation (Chapter IV). Furthermore, M-T7 functions early in infection to retard inflammatory cell migration into infected tissues, and the M-T7$^-$ myxoma virus mutant did not elicit as effective an inflammatory response within the primary site of inoculation as that observed upon infection with the M-T5$^-$ mutant virus.

Thus, we conclude that the M-T5 protein of myxoma virus functions as a critical virulence factor by preventing the abortive infection of rabbit lymphocytes, allowing for the replication and dissemination of the virus throughout the lymphoreticular system. Given the importance of how lymphotropism is regulated by viruses in general, and the observation that the M-T5 protein appears highly specific for lymphocytes, further biochemical analysis of this protein is warranted. Since the natural history of myxoma virus and its host has been well established (11, 26, 27), the myxomatosis model should prove useful to further our understanding of viral-host interactions, particularly in terms of how immune cells themselves respond to virus infection.

Table VI-1. Summary of percent identity and similarity between the amino acid sequences of myxoma virus M-T5 and several poxviral host range superfamily members.

identity / similarity	MYX T5	CPV CHOhr	VV B4R	VV K1L	VV C7L	VAR B6R	EV K1L	RPV SPI-1
MYX T5	—	19.7	23.9	20.9	12.5	25.3	20.5	20.1
CPV CHOhr	47.9	—	27.1	28.7	23.4	26.4	23.4	17.3
VV B4R	48.3	51.2	—	23.3	25.5	94.2	23.5	19.2
VV K1L	49.3	55.3	51.6	—	24.5	22.1	96.5	18.3
VV C7L	45.0	52.5	51.0	46.9	—	25.2	23.1	21.0
VAR B6R	49.1	50.2	96.2	48.9	51.0	—	23.5	18.1
EV K1L	48.8	51.4	51.6	97.9	46.9	45.8	—	18.7
RPV SPI-1	46.6	44.9	44.4	45.4	47.1	45.6	44.0	—

Table VI-2: Apoptosis in RL5 lymphocytes infected with the M-T5$^-$ mutant myxoma virus

Hours (pi)	% Positive Apoptotic Cells		
	mock	vMyx	vMyxT5$^-$
0	nd	nd	1
4	nd	nd	20
8	nd	nd	41
12	1	10	50

Percentage of infected RL5 cells determined to be postitive for apoptosis by flow cytometric analysis of fragmented DNA. pi, post infection; nd, not determined.

Table VI-3: Pathogenicity of M-T5$^-$ mutant myxoma virus in European rabbits

Day	vMyx (wild type)	vMyxT5$^-$ (M-T5$^-$ mutant)
0	Inoculation of 4 rabbits with 1000 pfu vMyx	Inoculation of 8 rabbits with 1000 pfu vMyxT5$^-$
4	Primary lesions at inoculation sites, approx. 2cm, raised, soft and red	Primary lesions at inoculation sites, approx. 1cm, hard
7	Gram negative bacterial infections of nasal and conjunctival mucosa; multiple secondary skin lesions on face and ears	No Gram negative infections or secondary lesions detected
10	Severe Gram negative bacterial infections in conjunctiva and respiratory tract; secondary skin lesions turning necrotic; all rabbits sacrificed due to increasing severity of symptoms	No Gram negative infections detected; single minute secondary lesion detected in one ear of one rabbit
14	---------	Full regression of primary lesions; rabbits immune to subsequent challenge with vMyx (wild type) infection

Table VI-4: Major histological differences between vMyxlac (M-T5$^+$) and vMyxlacT5$^-$ (M-T5$^-$) infection in European rabbits

	vMyxlac (wild-type)	vMyxlacT5$^-$ (M-T5$^-$ mutant)
Day 4	Primary sites[a] •inflammatory reaction with enhanced edema, presence of scattered heterophils •damaged epithelium	Primary sites[a] •inflammatory reaction with little to no edema, accumulation of heterophils, regeneration of damaged epithelium
	Secondary sites[b] •no detectable reaction	Secondary sites[b] •no detectable reaction
	Lymph nodes-mildly reactive	Lymph nodes-normal
	Spleen-mildly reactive	Spleen-normal
Day 7	Primary sites[a] •acute necrosis of epidermis •inflammation with edema and hemorrhage; necrosis of heterophils	Primary sites[a] •normal epithelium •inflammation without edema and hemorrhage; heterophils replaced by mononuclear cells
	Secondary sites[b] •thickened, disorganized epidermis •inflammatory reaction in dermis and subcutis; heterophils predominate	Secondary sites[b] •no detectable reaction
	Lymph nodes-loss of mature lymphocytes	Lymph nodes-normal
	Spleen-loss of mature lymphocytes	Spleen-normal
Day 10	Primary sites[a] •inflammatory reaction with necrosis, edema and hemorrhage; necrotic heterophils •degenerated epithelium	Primary sites[a] •resolution of infection •tissue sections normal
	Seconday sites[b] •thickened, disorganzied epidermis •inflammation with edema; heterophils and macrophages predominate	Seconday sites[b] •no detectable reaction
	Lymph nodes-loss of mature lymphocytes	Lymph nodes-mild reaction
	Spleen-necrosis of lymphocytes	Spleen-mild reaction

[a]primary site samples taken from site of inoculation in the thigh
[b]secondary site samples taken from ear, lip, nose and eyelid

Figure VI-1. Nucleotide sequence and corresponding amino acid sequence of the myxoma virus M-T5 gene. Sequencing of the complete M-T5 open reading frame in both orientations from genomic DNA was accomplished using a series of primers, as outlined in Chapter II. Nucleotide sequences flanking the M-T5 gene are also shown. The location of an ankyrin-like repeat within the amino acid sequence is highlighted with a solid line. GenBank accession number is U43201.

```
TGGGAACGTGTCGAGAATATGACGGAGTCGCGCAGGAATCCCATCTGCTGCGTCTACAACAACGCGCTGTACGTATTCGGAGGACGGACGAACTCGGCG
GAGAGCTATAACGGGTGGAAATGGCACCGCATCAACGACGTCTCTCTATACACGGAGTGTACGAACACGGCGTGTCCATTTTTTATACAACGGTGAAAA

                                M  D  L  Y  G  Y  V  S  C  T  P  R  I  R  H  D  V  L  D  G  L  L  N  23
AATAAGTGTAGTTGATATAACCCCGGACGAGGATGGATCTATACGGGTATGTGTCGTGTACGCCTCGTATCCGACACGATGTACTCGATGGTCTCTTGA

   V  Y  D  P  D  E  L  C  S  R  D  T  P  F  R  L  Y  L  T  R  Y  D  C  T  P  E  G  L  R  L  F  L  T  56
ACGTATACGACCCGGACGAACTATGTTCGCGGGATACCCCCTTTCGCCTTTACCTAACGAGATACGACTGTACCCCGGAGGGTTTACGTTTGTTTTTAA

   R  G  A  D  V  N  G  V  R  G  S  R  T  S  P  L  C  T  V  L  S  N  K  D  L  G  N  E  A  E  A  L  A  89
CACGAGGCGCGGACGTCAACGGAGTTCGTGGTCTCGTACGTCTCCTCTGTGTACGGTCCTATCCAATAAAGATTTGGGAAATGAGGCGGAGGCGTTGG

   K  Q  L  I  D  A  G  A  D  V  N  A  M  A  P  D  G  R  Y  P  L  L  C  L  L  E  N  D  R  I  N  T  A  122
CGAAACAGCTCATCGATGCGGGAGCCGACGTCAACGCGATGGCGCCAGATGGACGATATCCCTTGCTCTGTCTGCTGGAAAACGACCGTATTAATACGG

   R  F  V  R  Y  M  I  D  R  G  T  S  V  Y  V  R  G  T  D  G  Y  G  P  V  Q  T  Y  I  M  S  K  N  V  155
CGAGGTTTGTACGCTACATGATCGACCGAGGCACGTCCGTGTACGTTCGGGGAACGGACGGATACGGGCCCGTACAAACCTACATACATTCCAAGAACG

   V  L  D  T  L  R  E  L  V  R  A  G  A  T  V  M  D  P  D  K  T  Y  G  F  N  V  L  Q  C  Y  M  I  A  188
TGGTATTGGATACGTTACGAGAGTTGGTCCGCGCGGGAGCCACCGTACACGACCCAGACAAGACGTACGGGTTTAACGTCCTTCAATGTTACATGATCG

   H  V  R  S  S  N  V  Q  I  L  R  F  L  L  R  N  G  V  D  S  S  R  G  L  H  A  T  V  M  F  N  T  L  221
CCCACGTGCGTTCGTCGAACGTGCAGATTCTACGATTCCTTCTGCGTCACGGGGTCGATTCCTCCCGAGGATTACACGCGACCGTCATGTTTAACACGT

   E  R  K  I  S  M  G  V  F  N  R  K  V  L  D  F  I  F  T  Q  I  S  I  N  E  Q  N  S  L  D  F  T  P  254
TGGAACGTAAGATCAGCCACGGCGTCTTTAATCGAAAGGTGTTGGATTTTATCTTTACACAAATAAGCATAAACGAACAAAACTCTCTCGACTTTACAC

   I  N  Y  C  V  I  H  N  D  R  R  T  F  D  Y  L  L  E  R  G  A  D  P  N  V  V  N  F  L  G  N  S  C  287
CCATTAACTATTGCGTGATACACAACGACCGGCGAACGTTCGACTATCTGCTGGAGAGAGGAGCCGATCCCAACGTGGTGAACTTTCTGGGGAACTCTT

   L  D  L  A  V  L  N  G  N  K  Y  M  V  H  R  L  L  R  K  T  I  T  P  D  A  Y  T  R  A  L  N  V  V  320
GTCTAGACCTGGCCGTGTTAAACGGAAACAAGTACATGGTCCATCGTCTGCTACGAAAGACGATCACGCCGGACGCCTACACGAGGGCGTTGAACGTGG

   N  S  N  I  Y  S  I  K  S  Y  G  M  S  E  F  V  K  R  H  G  T  L  Y  K  A  L  I  R  S  F  V  K  D  353
TAAACTCTAACATTTACTCGATCAAGTCGTACGGCATGTCGGAGTTCGTAAAGCGACACGGAACCTTGTACAAAGCCCTCATTCGAAGCTTCGTAAAAG

   S  D  R  E  I  F  T  Y  V  H  I  Y  D  Y  F  R  E  F  V  D  E  C  I  R  E  R  D  A  M  K  A  D  V  386
ACTCGGATAGGGAGATCTTTACATACGTTCACATCTACGACTACTTTAGAGAGTTTGTGGACGAATGTATAAGGGAACGAGACGCCATGAAGGCGGACG

   L  D  A  V  S  V  F  D  T  A  F  G  L  V  A  R  P  R  W  K  H  V  R  I  L  S  K  Y  V  R  G  V  Y  419
TTCTGGACGCGGTCAGCGTGTTCGATACAGCGTTCGGTCTAGTCGCCAGGCCTCGTTGGAAACACGTTCGGATTCTCTCGAAGTACGTACGAGGCGTCT

   G  D  R  V  K  K  I  L  R  S  L  H  K  R  R  F  K  T  D  R  L  V  R  R  I  A  D  L  C  G  P  D  G  452
ACGGAGATCGAGTAAAGAAGATTCTCCGATCCCTACACAAGCGACGGTTCAAAACGGATCGTCTCGTTCGCCGTATCGCCGACCTGTGCGGACCCGACG

   L  W  T  R  L  P  V  E  V  R  Y  S  V  V  D  Y  L  T  D  D  E  I  H  D  L  .  V  K  I  H  A  •  483
GTCTATGGACCCGTCTTCCCGTAGAAGTCCGATACAGCGTCGTGGATTATCTGACGGATGACGAAATACACGATCTTTTTGTAAAGATACACGCGTAGG
AACTTTCTCGTATTATAAATGCTATCCTATTTGGTAGGTCCTATCCTATCGATTTGCTACTTCGTTATAGGACGATTGANCGGATTAGTCCAATACCTG
GGTCGGGAAAATGATA
```

Figure VI-2. Amino acid alignment between myxoma virus M-T5 and representative homologous poxviral proteins. Amino acid comparison between the myxoma virus M-T5, Shope fibroma virus S-T5, vaccinia virus VV-B4R and cowpox virus CHO-hr proteins. Identical residues between at least three of four sequences are highlighted by boxes. Gaps introduced to maintain optimal alignment are indicated by dots.

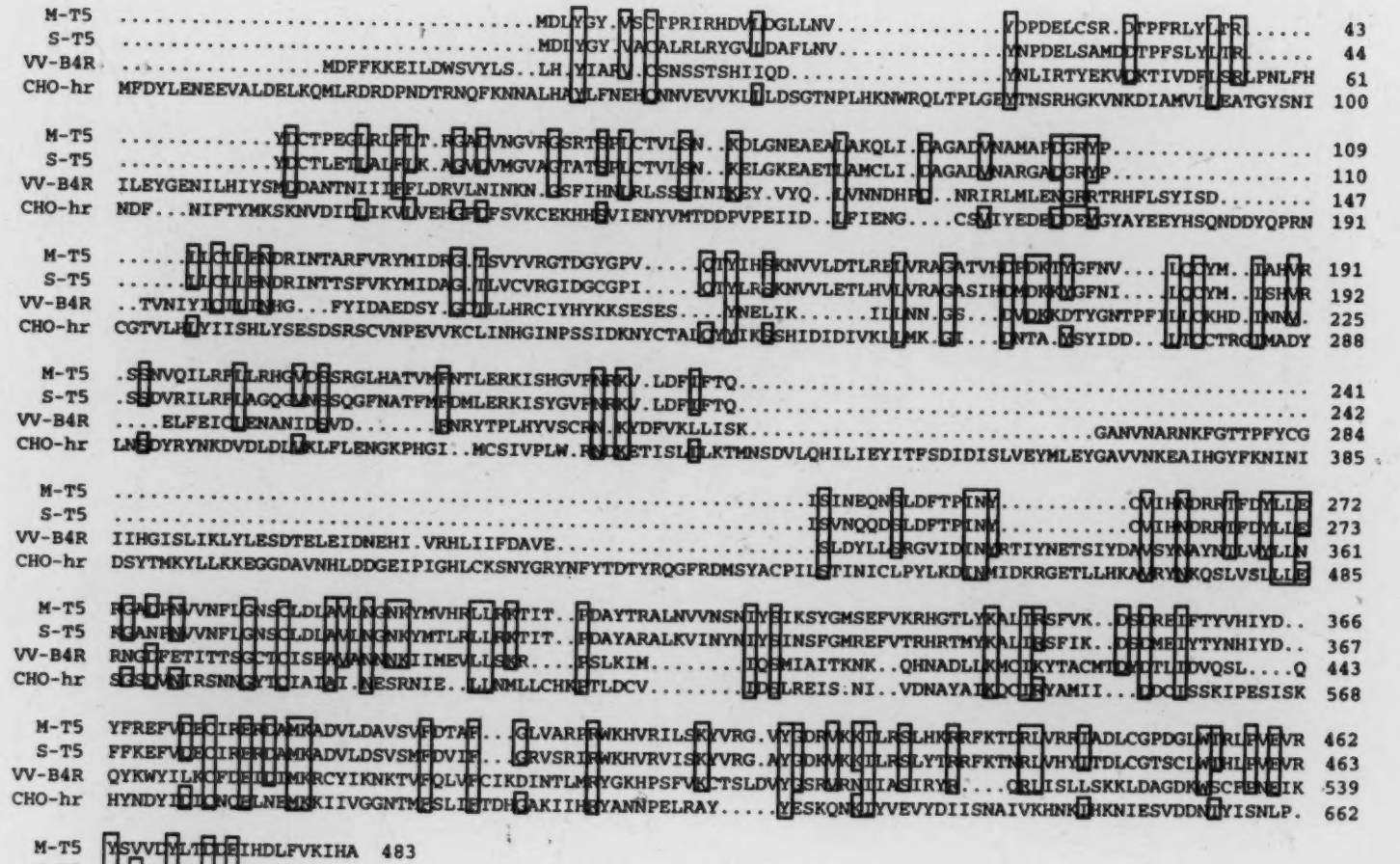
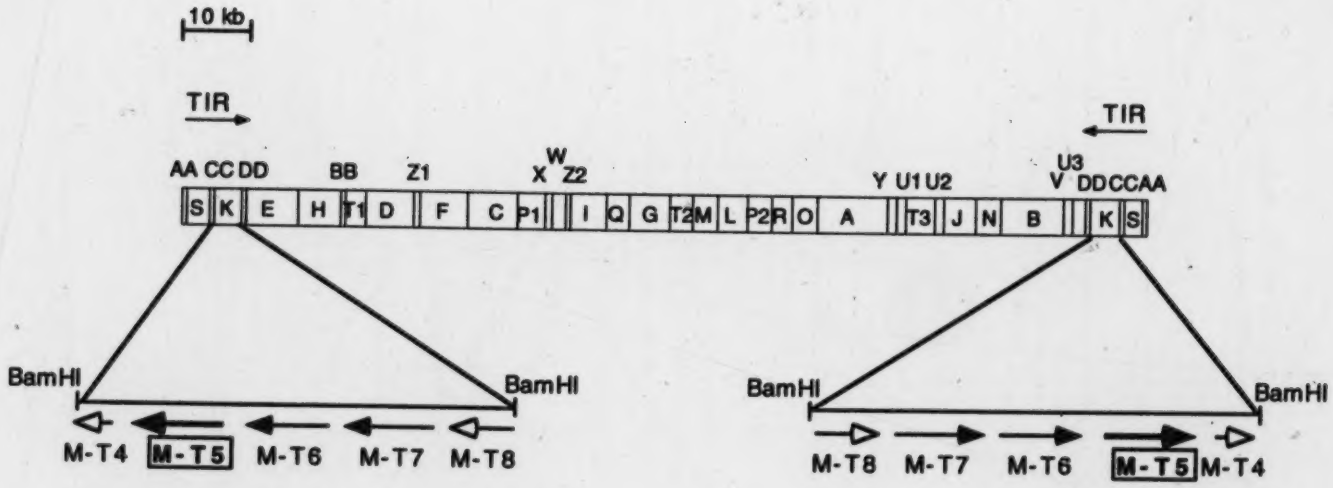


Figure VI-3. Construction of a recombinant myxoma virus containing a disrupted M-T5 gene. (A) Location of both copies of the M-T5 open reading frame within the terminal inverted repeat (TIR) region of the myxoma virus genome. The BamHI map of the myxoma virus genome (37) is illustrated. Filled arrows denote complete open reading frames within the BamHI K fragment whereas open arrows illustrate genes which flank this fragment. (B) Construction of M-T5$^-$ disruption viruses. The BamHI K fragment was cloned into pBluescript creating the plasmid pBamK. PCR amplification was used to generate the M-T5 terminal fragments M-T5L and M-T5R, which were engineered to contain complimentary ends that create an internal PstI site. These fragments were subsequently mixed, amplified, and cloned into pBluescript creating the plasmid pMT5del. Digestion with PstI and insertion of a PstI p7.5Ecogpt cassette was used to form the M-T5 disruption plasmid pMT5gpt, which was subsequently transfected into vMyx and vMyxlac infected cells to create vMyxT5$^-$ and vMyxlacT5$^-$ disruption viruses, respectively.

A.


10 kb
TIR
TIR
AA CC DD
BB
Z1
W
X
Z2
Y U1 U2
U3
V
DD CC AA
S K E H T1 D F C P1 I Q G T2 M L P2 R O A T3 J N B K S
BamHI
BamHI
BamHI
BamHI
M-T4
M-T5
M-T6
M-T7
M-T8
M-T8
M-T7
M-T6
M-T5
M-T4

B.

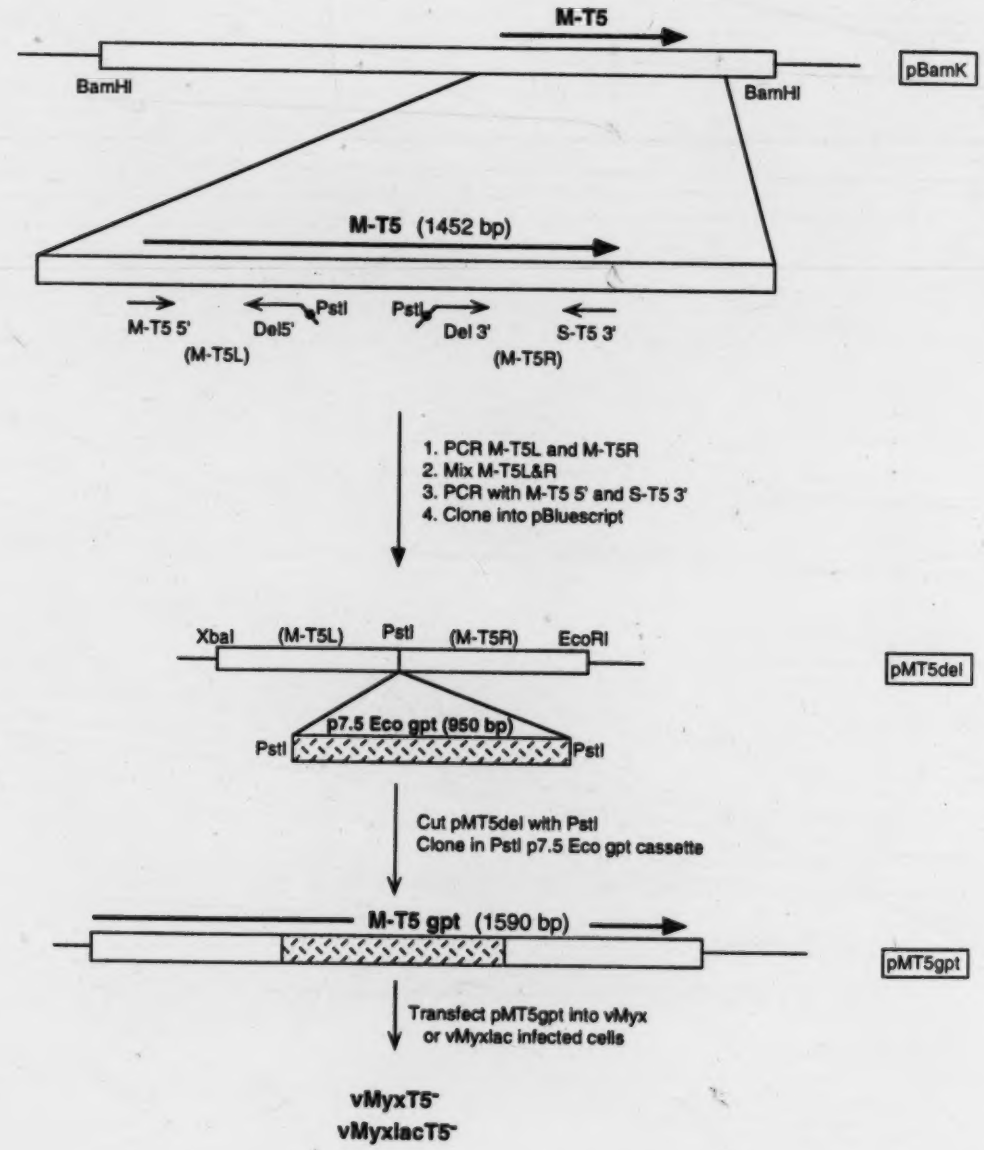

M-T5
BamHI
BamHI
pBamK
M-T5 (1452 bp)
PstI
PstI
M-T5 5'
Del5'
Del 3'
S-T5 3'
(M-T5L)
(M-T5R)
1. PCR M-T5L and M-T5R
2. Mix M-T5L&R
3. PCR with M-T5 5' and S-T5 3'
4. Clone into pBluescript
XbaI
(M-T5L)
PstI
(M-T5R)
EcoRI
pMT5del
p7.5 Eco gpt (950 bp)
PstI
PstI
Cut pMT5del with PstI
Clone in PstI p7.5 Eco gpt cassette
M-T5 gpt (1590 bp)
pMT5gpt
Transfect pMT5gpt into vMyx
or vMyxlac infected cells
vMyxT5-
vMyxlacT5-

Figure VI-4. Characterization of the M-T5 gene from myxoma virus M-T5 recombinants. Agarose gel electrophoresis of PCR products amplified using the M-T5 5' and S-T5 3' primers (refer to Figure VI-3b) demonstrate the difference in migration between wild-type M-T5 (lane 2) and the disrupted recombinant M-T5gpt (lane 3) derived from pBamK and pMT5gpt plasmid DNA, respectively. PCR analysis of viral DNA harvested from cells infected with vMyxT5$^-$ (lane 4), vMyxlacT5$^-$ (lane 5) and vMyxT5rev (lane 6) demonstrate the presence of either M-T5gpt or M-T5, indicating the purity of the recombinant viral stocks. Molecular weight markers of 8450, 5686, 2323 and 1371 bp corresponding to BstEII cut λ standards (lane 1) are indicated on the left.

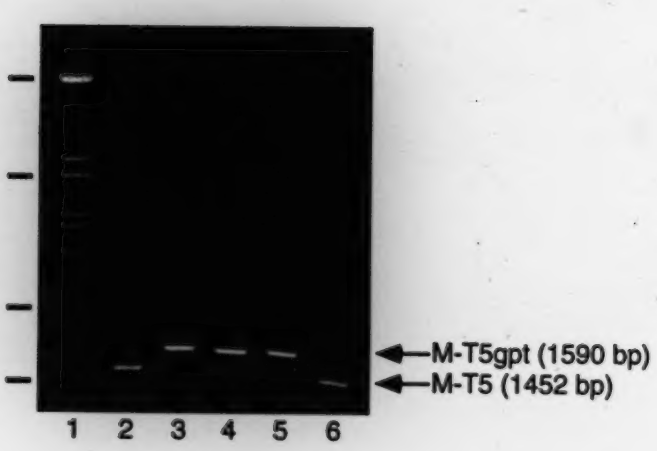
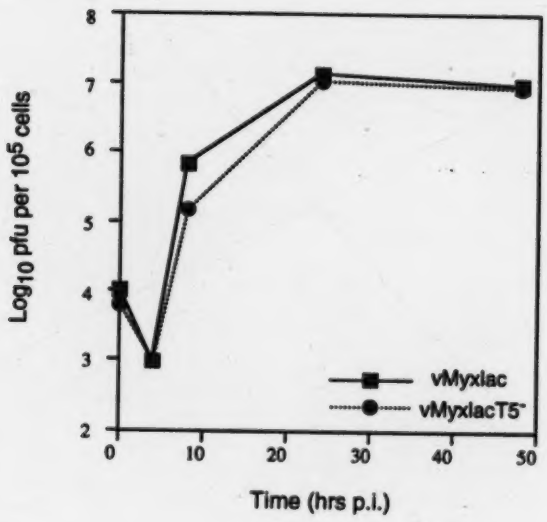

M-T5gpt (1590 bp)
M-T5 (1452 bp)
1 2 3 4 5 6

Figure VI-5. Single step growth analysis of wild type and M-T5$^-$ mutant myxoma viruses. Infection of (A) rabbit kidney fibroblast (RK13) cells and (B) rabbit CD4$^+$ T-lymphocyte (RL5) cells with vMyxlac and vMyxlacT5$^-$ at a multiplicity of infection of 5. Cells were harvested at the indicated times post-infection and infectious virus titred on RK13 cells. Each viral growth analysis was performed in triplicate, with the mean plotted.

A.


Log10 pfu per 10^5 cells
8
7
6
5
4
3
2
0
10
20
30
40
50
Time (hrs p.i.)
vMyxlac
vMyxlacT5-

B.

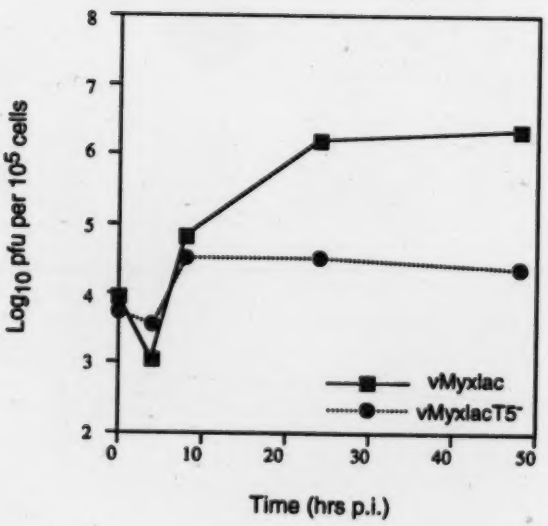
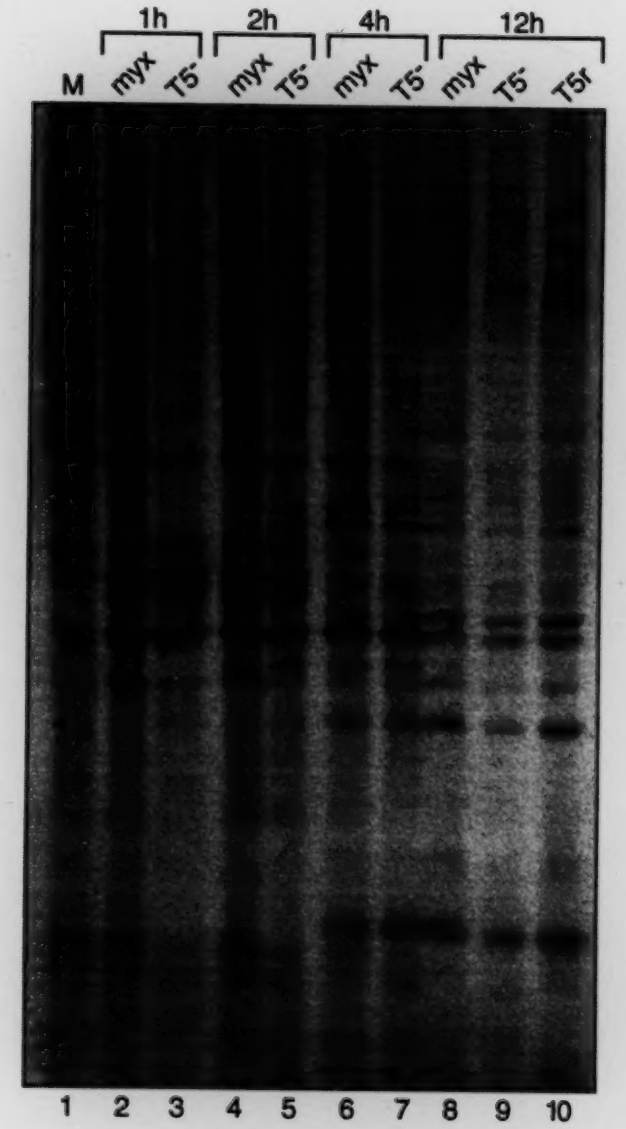
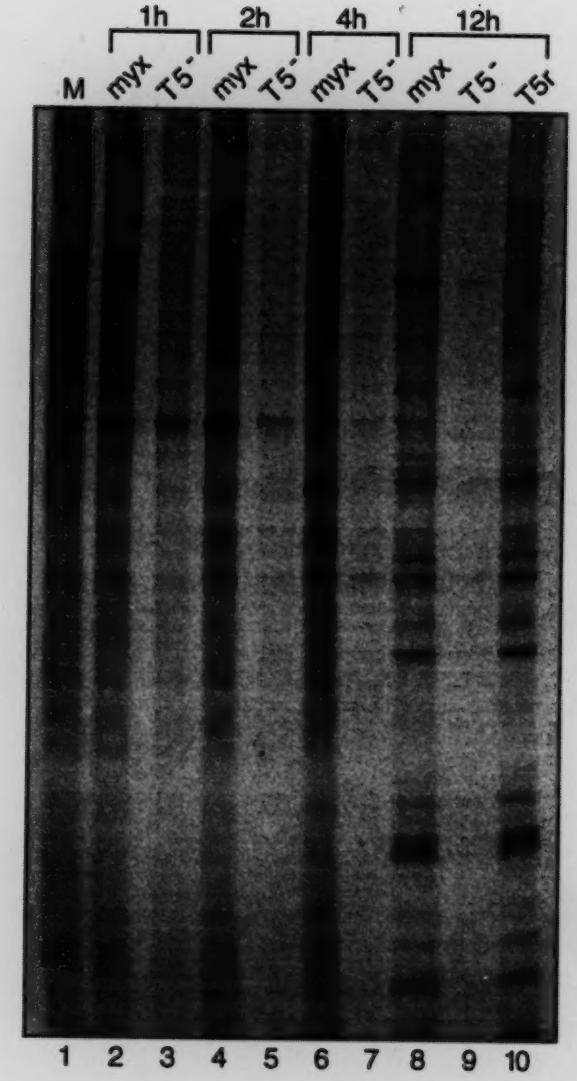

Log10 pfu per 10^5 cells
8
7
6
5
4
3
2
0
10
20
30
40
50
Time (hrs p.i.)
vMyxlac
vMyxlacT5-

Figure VI-6. Metabolic labeling of myxoma virus infected cells with [^{35}S]cysteine/methionine. RK13 fibroblasts (A) or RL5 lymphocytes (B) were either mock infected (M) or infected with vMyxlac (myx), vMyxlacT5$^-$ (T5$^-$) or vMyxT5rev (T5r), and labeled with 50 μCi of [^{35}S]cysteine/methionine for 30 min at the indicated times post-infection. Cells were harvested into SDS loading buffer, and analyzed by SDS-PAGE followed by autoradiography. (C) RL5 lymphocytes were either mock infected (M), or infected with vMyxlac (myx), vMyxlacT5$^-$ (T5$^-$), vMyx-T2$^-$ (T2$^-$), vMyx-M11L$^-$ (M11L$^-$), vMyx-T7$^-$ (T7$^-$) or vMyx-SERP1$^-$ (SERP1$^-$), harvested at the indicated times post-infection, and treated as in (A) and (B). Dashes indicate the positions of molecular weight markers, 101, 83, 50.6, 35.5, 29.1, and 20.9 kDa respectively.

A.



B.



C.

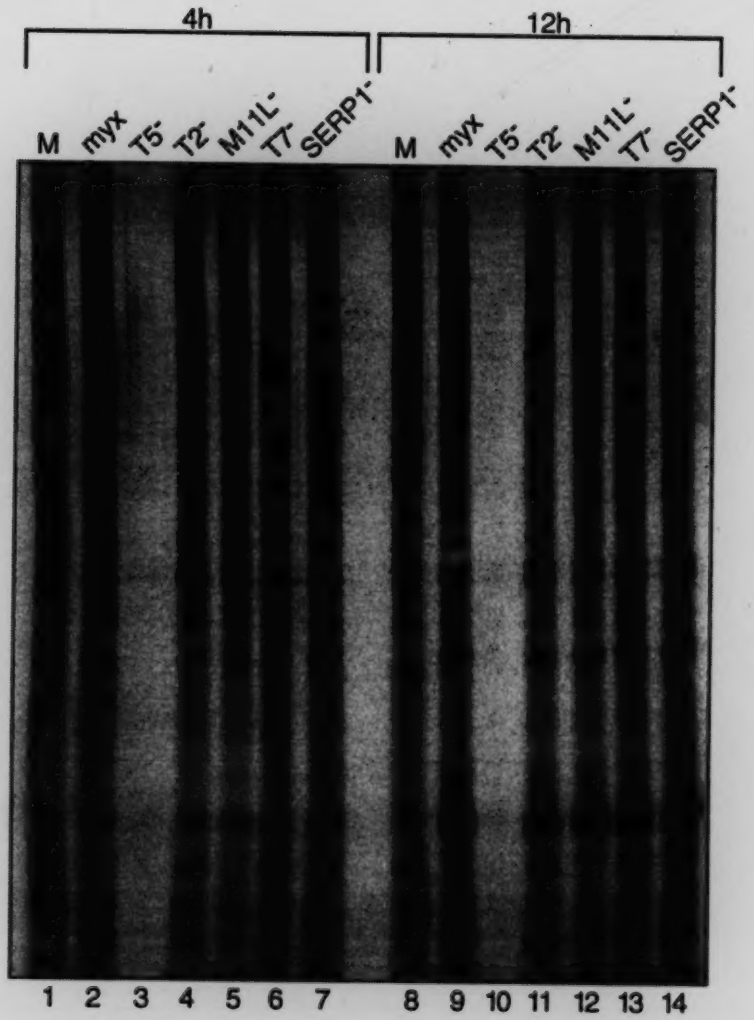

4h
12h
M
myx
T5-
T2-
M11L-
T7-
SERP1-
M
myx
T5-
T2-
M11L-
T7-
SERP1-
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure VI-7. Apoptosis in RL5 lymphocytes infected with the myxoma M-T5$^-$ mutant virus. Agarose gel electrophoresis of RL5 cells either mock infected (M) or infected with vMyxlacT5$^-$ (T5$^-$), vMyxlac (myx) or vMyxT5rev (T5r). At the indicated times post-infection, cells were harvested, digested with proteinase K and RNase, ethanol precipitated and subjected to agarose gel electrophoresis. Molecular weight markers of 8450, 3675, 1929 and 702 bp corresponding to BstEII cut λ standards (lane 1) are indicated on the left.

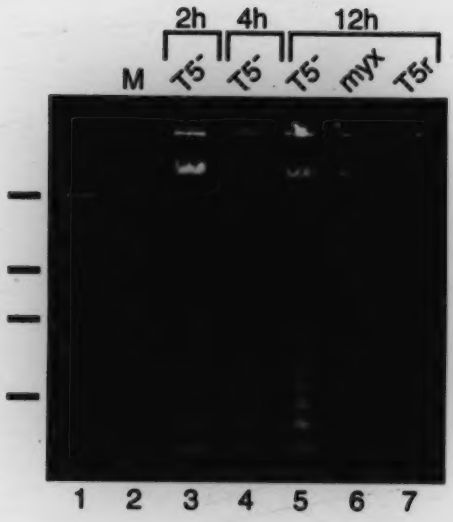
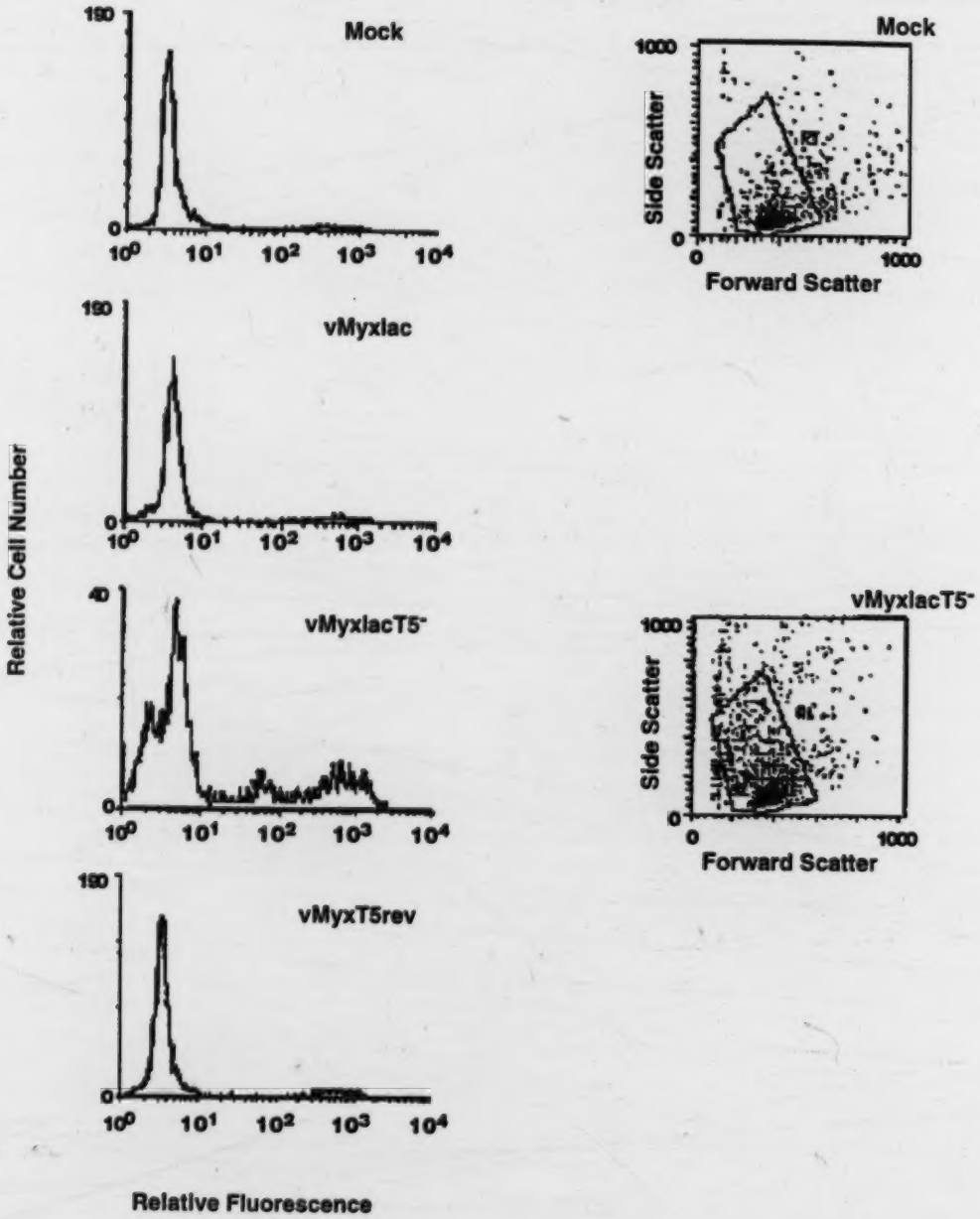

2h
4h
12h
M
T5-
T5-
T5-
myx
T5r
1 2 3 4 5 6 7

Figure VI-8. Flow cytometry analysis of infected rabbit peripheral blood mononuclear cells. Rabbit peripheral blood mononuclear leukocytes were separated into non-adherent (A) and adherent (B) fractions, enriching for lymphocytes and monocytes, respectively. Cells were then infected with the indicated viruses, harvested 12 hours post-infection, and subjected to the TUNEL method of detecting dNTP-addition to 3'-hydroxyl ends of fragmented DNA. The cell scatter profiles of peripheral blood cells either mock infected or infected with vMyxlacT5$^-$ are shown beside their corresponding fluorescence profile. The scatter profile of vMyxlac and vMyxT5rev infected cells were indistinguishable from mock infected cells. Flow cytometry performed by S. F. Lee.

A.


Mock
vMyxlac
vMyxlacT5⁻
vMyxT5rev
Relative Cell Number
Relative Fluorescence
Side Scatter
Forward Scatter

B.

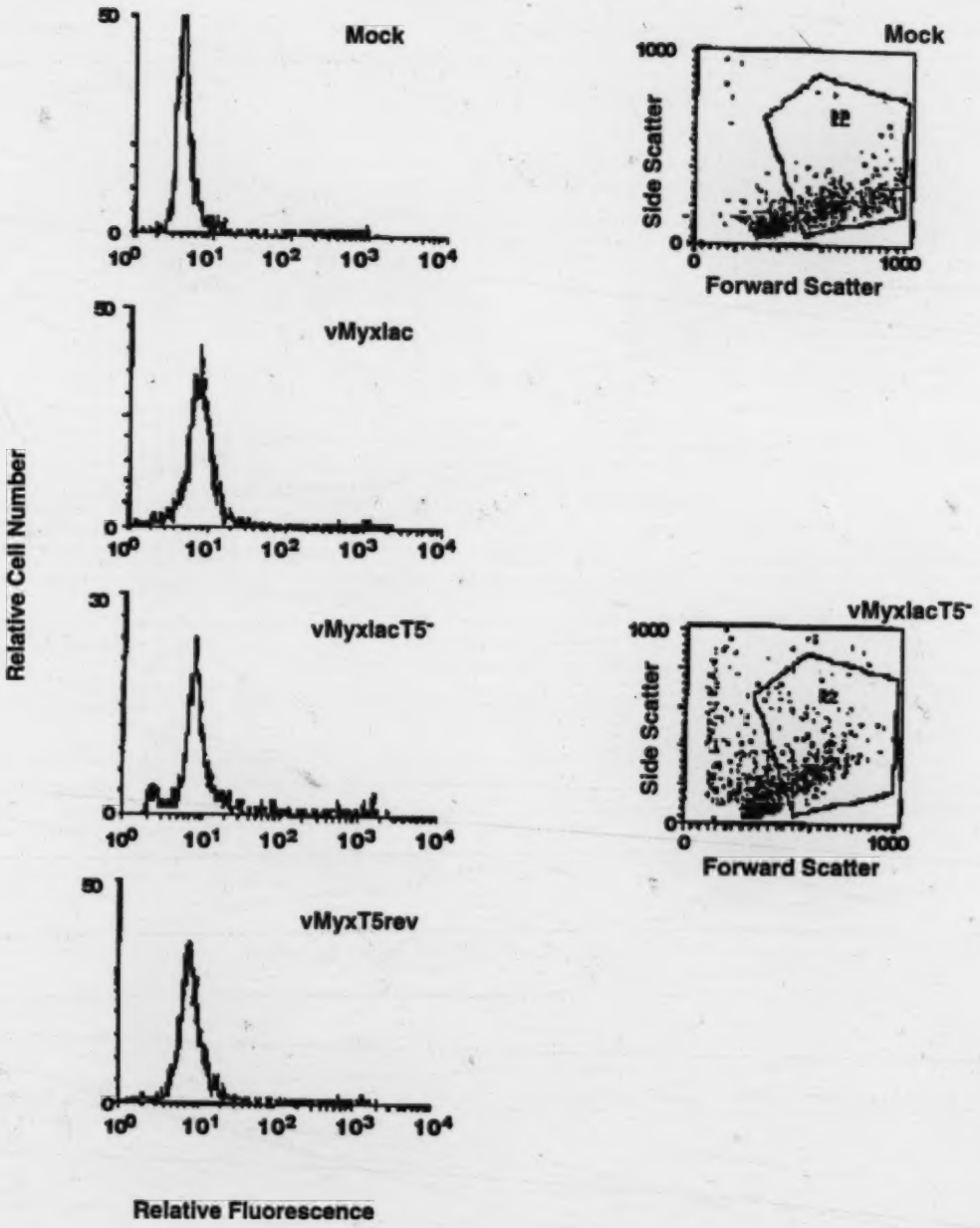
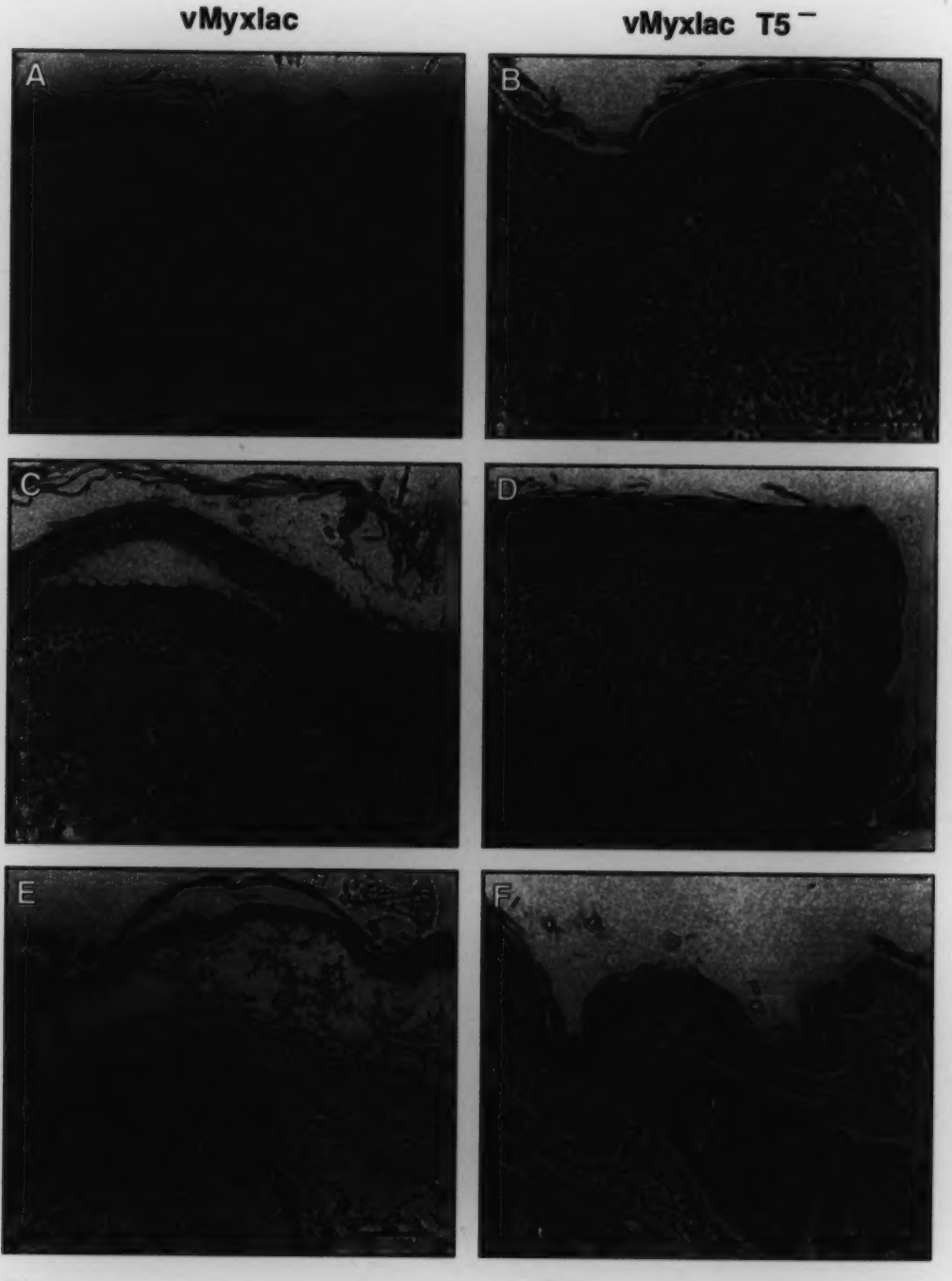

Mock
vMyxlac
vMyxlacT5-
vMyxT5rev
Relative Cell Number
Relative Fluorescence
Side Scatter
Forward Scatter
R2

Figure VI-9. Primary inoculation site tissue sections. Tissue sections from the primary site of infection with vMyxlac or vMyxlacT5$^-$ viruses harvested at day 4 (A and B, respectively), day 7 (C and D, respectively), and day 10 (E and F, respectively) post-inoculation. Tissue sections were harvested and stained with hematoxylin and eosin. 100μm scale is shown. Histological analyses performed by Dr. P. Nation.


vMyxlac
vMyxlac T5⁻
A
B
C
D
E
F

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CHAPTER VII

Myxoma Virus and Shope Fibroma Virus Encode Dual-Specific Tyrosine/Serine Phosphatases Which are Essential for Virus Viability[1]

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Introduction

Reversible phosphorylation of proteins is a well established mechanism in controlling eukaryotic intracellular events (14). Overall cellular phosphoprotein levels are controlled by a dynamic balance between protein kinases and protein phosphatases (6, 7, 22, 23). The vast majority of kinases and phosphatases act on serine and threonine residues, and until 1980 these two residues were the only identified naturally occuring phosphoamino acids (24). Studies with Rous sarcoma virus drew attention to tyrosine phosphorylation when it was shown that v-src, the virus gene product responsible for transformation, encoded a cell derived protein tyrosine kinase (24). Even though tyrosine phosphorylation accounts for less than 1% of the total protein phosphoamino acid content in a normal cell, tyrosyl phosphorylated proteins have been implicated as critical regulators of many important signal transduction cascades. Protein tyrosine phosphatases have recently been recognized as important counterparts to protein tyrosine kinases in the regulation of tyrosine phosphorylation of cellular proteins (6, 13, 28, 29, 49). Tyrosine phosphatases are as structurally diverse and complex as their cognate kinases, and can be classified into two major groups: transmembrane or receptor-like, and cytosolic or nonreceptor-like. Both classes of tyrosine phosphatases contain one or two homologous catalytic domains where the active site cysteinyl residue is located within a conserved sequence motif (20). Thus, both tyrosine kinases and phosphatases co-ordinate in the regulation of such diverse activities as proliferation, differentiation, and transformation (28, and references therein).

The ability to rapidly alter cellular function is also one attribute of poxviruses. Due to their cytoplasmic localization of replication, poxviruses encode most of the enzymes required for virus replication and transcription.

Among the various poxviral encoded enzymes are numerous regulatory enzymes, such as protein kinases (1, 30, 31, 44) and protein phosphatases (17, 33). The first poxviral tyrosine phosphatase to be characterized was a cytosolic phosphatase encoded by the vaccinia virus H1L open reading frame, often refered to as VH1 (17). Unlike all previously characterized tyrosine phosphatases which contain strict specificity for phosphotyrosyl residues, H1L was shown to dephosphorylate both phosphorylated tyrosyl and seryl residues, *in vitro*. Subsequently, other phosphatases with similar dual specificity have been characterized from other systems (26, 27).

Poxviruses encode a plethora of proteins which counteract host defence mechanisms, including such diverse targets as complement, growth factors, cytokines, MHC-restricted antigen presentation and regulation of both B cell and T cell activation (reviewed in 5, 37, 40, 45). It is conceivable that a poxvirus encoded phosphatase could perturb a variety of functions, ranging from the replication of the viral genome to the disruption of cellular signalling pathways critical for the host immune response. Here, we report the identification of a cytosolic tyrosine phosphatase expressed by the *Leporipoxviruses* SFV and myxoma virus, the latter of which is best known for its ability to cause lethal myxomatosis in the European rabbit (12, 35, 36). We have investigated the properties of the myxoma tyrosine phosphatase, including genomic localization, expression profile, substrate specificity, and the requirement of phosphatase for virus viability in cultured cells.

Results

Genomic Localization of Putative SFV and Myxoma Phosphatase Genes.

Exploratory sequencing of cloned SFV BamHI fragments (50) led to the fortuitous discovery of a putative phosphatase sequence motif in the BamHI-M fragment. Sequencing of the entire 2.7 kb BamHI-M fragment revealed that the SFV M1L open reading frame was homologous to that of vaccinia H1L, which encodes for a phosphatase (17), often refered to as VH1. Identification of the myxoma phosphatase gene was assisted by the high degree of homology between the SFV and myxoma virus genomes (35). Using PCR primers directed against SFV M1L, a 0.5 kb amplified product was obtained using myxoma genomic DNA. Southern blot analysis using this amplified product as a probe localized the open reading frame within the BamHI-I fragment of the myxoma genome (38) (data not shown). The myxoma phosphatase gene was sequenced from the cloned BamHI-I fragment of myxoma DNA and designated I1L, since it is the first complete open reading frame within the BamHI-I fragment, and is oriented towards the left end. Figure VII-1 outlines the genomic localization of the myxoma and SFV phosphatase open reading frames.

The SFV BamHI-M and myxoma BamHI-I fragments are both located approximately 64kb from their respective left TIRs, demonstrating similar genomic localizations (Figure VII-1). Previous studies suggested that the TIRs of SFV and myxoma virus were also quite similar (46, 47). Comparison of the SFV M1L and myxoma I1L DNA sequences demonstrated an identity of 91.9% (Figure VII-2) and the conserved location of portions of the two flanking open reading frames indicates a common evolutionary origin for SFV and myxoma virus (refer to Figure VII-1). To the right of M1L of SFV and I1L of myxoma, are

M2R and I2R, respectively. M2R and I2R were identified as homologs to the similarly located H2R open reading frame of vaccinia virus (16), and I2R of variola virus (33). To the left of M1L of SFV and I1L of myxoma are sequences which are homologous to the C-terminal portion of RP0147, the largest RNA polymerase subunit encoded by the J6R open reading frame of vaccinia virus (4), which is also well conserved with the variola L6R open reading frame (33). Thus, we found that the location and orientation of the two *Leporipoxvirus* phosphatase genes are closely correlated, and both contain significant similarities to the genomic organization of the two *Orthopoxvirus* phosphatase genes studied to date.

Comparison of the Poxviral Phosphatase Amino Acid Sequences.

Database searching demonstrated that the highest degree of homology at the amino acid level to myxoma I1L and SFV M1L was to the vaccinia H1L tyr/ser phosphatase (17), and to the variola virus I1L phosphatase (33, 39). Alignment of these four poxviral phosphatases demonstrates the extent of this homology (Figure VII-3). Spanning the entire length of the polypeptides, 62 % of the amino acids were found to be identical in all four proteins. As well, only five amino acids differ between the SFV and myxoma proteins, while there are four amino acid differences between the vaccinia and variola proteins. All four viral phosphatases contain the conserved signature sequence motif (I/V)HCXAGXXR(S/T)G (13), characteristically located within the catalytic domain of tyrosine phosphatases. Within this motif is the active site cysteine, which is essential for tyrosine phosphatase function (17, 41). When compared to phosphatases from *Yersinia* (19), *S. cerevisiae* (18), and *S. pombe* (15), the highest degree of homology was found within the signature sequence motif

(data not shown). Thus, the poxviral tyrosine phosphatases constitute proteins which are highly conserved, both within and between different genera.

Myxoma I1L is Expressed as a Late Gene Product.

Poxviral promoters have been characterized as early, intermediate, and late (8, 9, 25, 48), and the four poxviral phosphatases in Figure VII-3 all contain the typical TAAAT motif characteristic of late poxvirus promoters. The TAAAT motif of the myxoma and SFV phosphatase genes overlaps with the putative initiating ATG, also a typical feature of late promoters. Northern blot analysis was used to examine the expression of the I1L gene in myxoma infected cells. As seen in Figure VII-4, I1L transcripts are only seen at late times, following the commencement of viral DNA synthesis, predominantly beginning 7 hours post-infection (lane 4). Also, the heterogeneous size of the viral transcripts is another feature commonly observed for late poxviral mRNAs. When the same viral RNA samples were subjected to Northern blot analysis with a probe to a control early gene, the myxoma M-T7 gene, single transcripts were observed only at the 2 hr and 4 hr post-infection samples (data not shown). Thus, we conclude that the I1L gene of myxoma is expressed as a classical late viral gene.

Myxoma I1L Protein is a Functional Dual Specific Tyrosine/Serine Phosphatase.

Given the similarity between the SFV and myxoma phosphatases at the amino acid level (97% identity), all subsequent experiments were carried out with the myxoma protein. To demonstrate that the myxoma I1L gene encodes a functional phosphatase, the protein was expressed as a GST fusion protein, in parallel with the vaccinia H1L protein and a myxoma mutant (I1L-C110S), in

which the putative active site cysteine was changed to a serine. This cysteine to serine mutation at amino acid 110 was previously shown to abolish all phosphatase activity of vaccinia H1L (17). As seen in Figure VII-5a and b, both myxoma I1L and vaccinia H1L are capable of hydrolyzing p-NPP, a chromogenic substrate structurally related to phosphotyrosine, and dephosphorylating tyrosine-phosphorylated myelin basic protein, respectively. Loss of the active site cysteine in I1L-C110S completely abolished the ability of the mutant myxoma I1L enzyme to dephosphorylate both substrates.

Most tyrosine phosphatases possess strict specificity for tyrosyl-phosphorylated residues (6). However, the vaccinia phosphatase H1L was shown to possess specificity for phosphoserine as well as phosphotyrosine (17). Given the substantial differences between the biological characteristics of vaccinia and myxoma, it was important to determine if myxoma I1L also possessed dual substrate specificity. As shown in Figure VII-5c and d, the vaccinia and myxoma phosphatases each dephosphorylated phospho-serine residues on both casein and myelin basic protein substrates. Once again, mutation of the active site cysteine 110 to serine in myxoma I1L totally abolished its enzymatic activity. Neither the vaccinia nor the myxoma enzyme was capable of dephosphorylating serine-phosphorylated phosphorylaseA (data not shown). Although myxoma I1L appears to have a higher specific activity for the dephosphorylation of tyrosine-phosphorylated substrates than does vaccinia H1L, and the two enzymes apparently have the opposite affinities for serine-phosphorylated substrates (Figure VII-5), it must be noted that both enzymes are GST fusion proteins expressed from a bacterial system, and the substrates used for these assays are useful only as *in vitro* targets. Thus, the biological preferences of these two viral phosphatases for physiologically relevant phosphorylated substrates remains to be determined.

Myxoma I1L is an Essential Gene.

In order to characterize the requirement for the myxoma dual specific phosphatase *in vivo*, we attempted to construct a mutant virus containing a disrupted I1L open reading frame, by homologous recombination. An I1L disruption plasmid vector was constructed by replacing 178 nucleotides of myxoma I1L coding sequences with a 3.15kb BglII p11-LacZ cassette (Figure VII-6a). Both orientations of p11-LacZ were constructed and tested for the ability to generate phosphatase-minus viruses, but were found to give identical results (data not shown). Recombinant viruses were selected for by plating under agarose containing X-gal, which would cause recombinant viruses that express β-galactosidase to stain blue. Five rounds of plaque purification continued to generate blue plaques that contained mixtures of wild type and mutant myxoma viruses but failed to produce virus isolates possessing only the disrupted copy of I1L (Figure VII-6b). All blue virus plaques at each round tested were strictly heterogeneous in nature, containing variable ratios of viruses possessing the wild-type allele in mixtures with the disrupted phosphatase gene. We conclude that since viruses containing the disrupted I1L gene could not propagate without the presence of a wild-type complementing gene product, then the myxoma phosphatase protein must be required for viral propagation. In blue plaques harboring both the mutant and wild-type viruses, sufficient wild-type phosphatase must therefore be present in doubly infected cells to permit propagation of the mutant phosphatase disruption virus, and thus maintain the appearance of blue plaques. To confirm these findings, PCR analysis was routinely utilized, using myxoma I1L primers, and primers specific for the LacZ gene to demonstrate that individual blue plaques which contained copies of virus genomes bearing the disrupted phosphatase gene also always harbored wild-type parental virus with the full

length phosphatase gene (Figure VII-6b). Thus we conclude that expression of I1L must be essential for viral propagation in tissue culture.

Discussion

Protein tyrosine phosphatases are now recognized as important regulatory enzymes, which in concert with protein tyrosine kinases regulate protein phosphorylation within a cell. Tyrosine phosphatases, whether receptor-like or soluble, generally possess a common catalytic domain, where the active site cysteine is located within the signature sequence motif (V/I)HCXAGXXR(S/T)G (13). Here, we have characterized the tyrosine phosphatases encoded by the *Leporipoxviruses* SFV and myxoma virus, which both contain the characteristic signature sequence motif, and which, at least in the case of myxoma, appears to be essential for virus viability. We have also characterized the specificity of the myxoma enzyme *in vitro*.

The poxviral phosphatases belong to a relatively new sub-family of soluble tyrosine phosphatases which, aside from the signature sequence motif, contain little similarity to other soluble tyrosine phosphatases. The phosphatases encoded by poxviruses are much smaller than other characterized cellular tyrosine phosphatases, which typically contain catalytic domains larger than the entire viral phosphatase proteins. Aside from the conserved tyrosine phosphatase signature sequence motif, the poxviral phosphatases do not contain any of the diverse protein sequences which appear to localize intracellular tyrosine phosphatases to defined subcellular compartments (34). Also, while most of the non-viral tyrosine phosphatases have been characterized as maintaining strict specificity for phosphotyrosyl residues, the poxviral enzymes have the ability of dephosphorylating both phosphotyrosyl and phosphoseryl residues *in vitro*.

Phosphatases encoded by poxviruses, including the myxoma I1L protein, are extremely intriguing when considering their potential biological functions. While the myxoma I1L phosphatase is characterized as a member of the

tyrosine phosphatase family due to the presence of the characteristic signature sequence motif, we have shown it to possess dual specificity *in vitro* to both phosphotyrosyl and phosphoseryl residues, similar to that of the vaccinia H1L phosphatase (17). Serine/threonine phosphatases generally exhibit broad and overlapping substrate specificities, and are known to function in glycogen and hepatic metabolism, protein synthesis, oocyte maturation, and muscle contractility (reviewed in 7). Tyrosine phosphatases, on the other hand, have been implicated in signal transduction, tumor suppression, cellular proliferation and differentiation control (reviewed in 28). Dual specific phosphatases, depending on their promiscuity, could thus potentially function in a wide variety of capacities.

In this report, we demonstrate for the first time the definitive importance of the poxviral phosphatases by showing that myxoma I1L is essential to virus viability in tissue culture. The inability to segregate myxoma virus isolates containing a disrupted copy of the I1L open reading frame from wild-type helper virus indicates that the myxoma phosphatase plays a crucial role in the viral life cycle. With respect to the poxviral life cycle, a virus encoded phosphatase such as myxoma I1L could function in viral processes obligatory for replication and propagation, in nonessential events that govern virulence, host-range, etc., or possibly in both classes of activities. In general, viral open reading frames which encode proteins that function to catalyze essential features of viral replication are clustered at central regions of the genome, are conserved among poxviruses, and are essential for basic virus survival (5, 45). Virus genes critical for pathogenesis and host-virus interactions, however, typically map towards the termini of the genome, show a high degree of variability among poxviruses, and are non-essential for viral replication in tissue culture (43). The open reading frames which encode for all the known poxviral

phosphatases are located within the central portion of the virus genome, adjacent to another essential virus gene, rpo147. Homologous phosphatase genes have also been reported in the central regions of eight other *Orthopoxviruses*, and a related gene has also been detected in the baculovirus *Autographa californica* (21). We believe that due to the high degree of similarity in both genomic localization and orientation, and in amino acid sequence, the other poxviral phosphatases are also essential enzymes. In addition, the vaccinia H1L phosphatase has recently been reported to be packaged into virions and required for early virus gene expression *in vivo* (32), further attesting to the essentiallity of this gene family for virus viability.

However, it is entirely possible that the myxoma phosphatase also functions directly in the progression of myxomatosis in rabbits, and that other poxviral phosphatases function in pathogenesis as well. The YOP2b phosphatase, which is expressed from the yopH gene in three different species of the *Yersinia* genus, has been shown to be essential for the virulence of this pathogen (19). YOP2b functions by dephosphorylating at least three major host proteins, whose roles in pathogenesis remain to be determined (3). Since *Yersinia* and poxvirus infections each radically affect the host cell macromolecular synthesis, it is feasible to postulate that the essential YOP2b and myxoma phosphatase proteins are key players in modulating host-protein phosphorylation. Furthermore, cdc25, which is a dual specificity tyrosine phosphatase structurally related to the poxviral phosphatases, is known to perform a critical regulatory function in the yeast cell cycle (11, 15). The *S. pombe* cdc25 protein functions to activate the protein kinase $p34^{cdc2}$ by dephosphorylating Tyr15 in the ATP-binding site, and ultimately drives the cell into mitosis. Poxviruses are known to be relatively S-phase independent and replicate in both quiescent and proliferating host cells, and hence there would

be a selective advantage to the virus specifically modulating the cell cycle machinery of the infected cell in situations of host cell quiescence.

Recently, the crystal structure of human PTP1B was solved, allowing specific predictions to be made on how tyrosine phosphatases interact with target substrates (2). The catalytic site of PTP1B was found to be located within a crevice, the base of which comprises the signature catalytic sequence motif containing the active site cysteine. Tyrosine side chains easily fit into the crevice, allowing contact between the phosphate moiety and the catalytic cysteine at the crevice base, whereas serine and threonine side chains are predicted to be too small to reach the base of the crevice, thus disallowing dephosphorylation of phosphoseryl and phosphothreonyl residues. While this observation produces a rational for the strict phosphotyrosyl specificity of most cellular tyrosine phosphatases, it does not explain how the dual specific poxviral phosphatases function. It has been shown for myxoma I1L (this thesis) and vaccinia H1L (17) using mutants in the active site cysteine, that this residue is required for dephosphorylation of both phosphotyrosyl and phosphoseryl residues. We predict that even though the dual specificity poxvirus phosphatases contain the conserved tyrosine catalytic motif, the three dimensional structures of the viral enzymes may have diverged sufficiently from the larger cellular enzymes to allow greater range of access by phosphorylated substrates.

Many critical questions remain as to which biological substrates the poxviral phosphatases recognize (42). Given the essentiallity of the myxoma I1L phosphatase for virus viability, it seems likely that some of the phosphoprotein substrates are of virus origin. The possibility that cellular substrates, particularly those such as cell cycle regulators or phosphorylated

intermediates that transduce signals from anti-viral cytokines such as TNF or interferon, are targets is particularly intriguing and merits further investigation.

Figure VII-1. Genomic localizations of the phosphatase open reading frames in myxoma virus and Shope fibroma virus. BamHI maps of myxoma (38) and Shope fibroma (10) viruses are shown, oriented in the same direction as has been standardized for vaccinia virus (16). The 6.4kb myxoma BamHI-I fragment and the 2.7kb SFV BamHI-M fragment have been expanded. The entire SFV BamHI-M fragment has been sequenced (solid arrows), whereas portions of the myxoma BamHI-I fragment remain to be sequenced (dotted arrows). The phosphatase open reading frames in myxoma and SFV are named I1L and M1L, respectively. I2R and M2R have closely related homologues similarly positioned in the genomes of vaccinia and variola. (RP0147) indicates close similarity with the C-terminal portion of the largest vaccinia RNA polymerase subunit, RP0147. Abbreviations: B=BamHI, TIR=terminal inverted repeat.

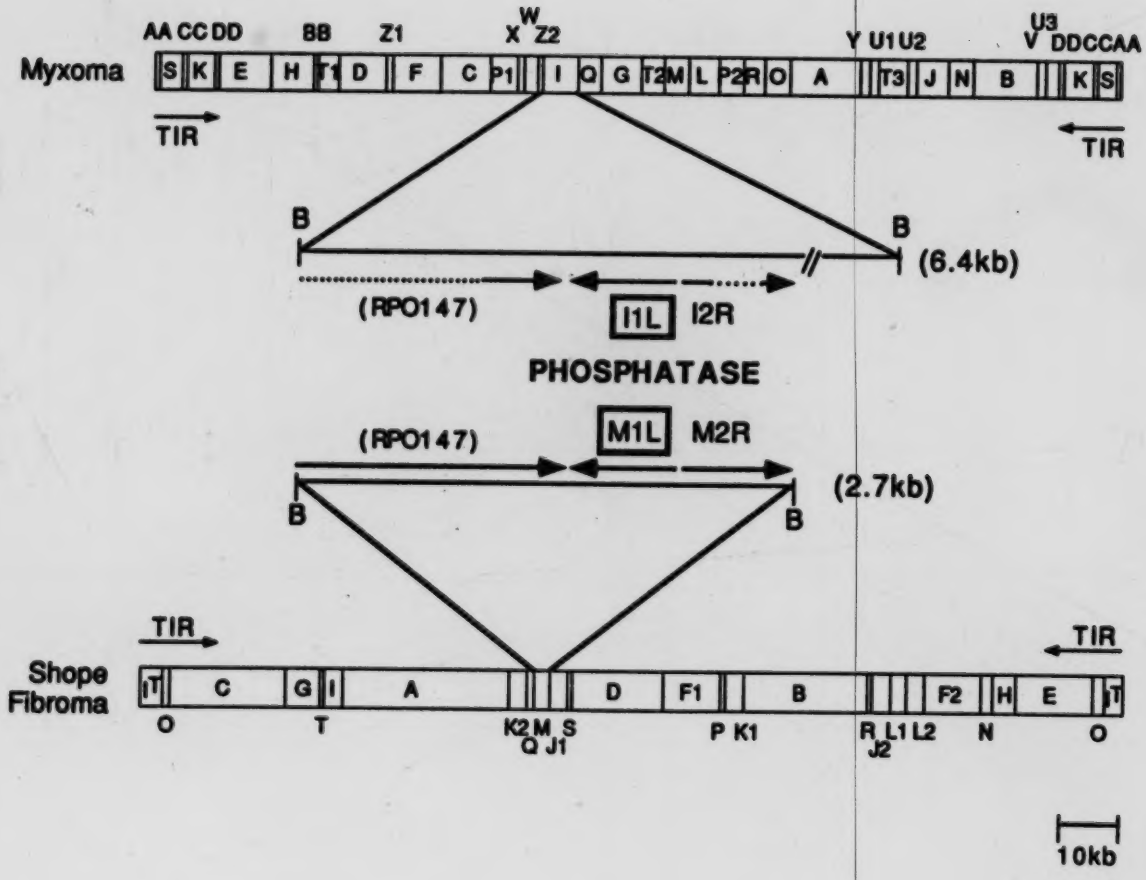

Myxoma
AA CC DD
BB
Z1
W
X Z2
Y U1 U2
U3
V DDCCAA
S K E H T1 D F C P1 I O G T2 M L P2 R O A T3 J N B K S
TIR
TIR
B
B
(6.4kb)
(RPO147)
I1L
I2R
PHOSPHATASE
(RPO147)
M1L
M2R
(2.7kb)
B
B
TIR
TIR
Shope
Fibroma
T C G I A D F1 B F2 H E T
O
T
K2 M S
Q J1
P K1
R L1 L2
J2
N
O
10kb

Figure VII-2. Alignment of myxoma and SFV protein tyrosine phosphatase (PTPase) open reading frames. Sequence alignment of the myxoma I1L open reading frame with the SFV M1L open reading frame. Restriction enzyme digestions mapped the 5' end of the myxoma I1L open reading frame approximately 2.2kb from the left end of the myxoma BamHI-I fragment, as oriented in Figure VII-1. The SFV M1L open reading frame begins 424 nucleotides from the right end of the SFV BamHI-M fragment, and ends 1744 nucleotides from the left end of the SFV BamHI-M fragment, as oriented in Figure VII-1. The start and stop codons are boxed, while the putative late promoter element, TAAAT, is underlined. Filled arrow indicates the start of the phosphatase open reading frames, while the open arrow indicates the start of the myxoma I2R and SFV M2R open reading frames (refer to Figure VII-1). Vertical lines depict identical nucleotides among the two sequences, and dots within the sequence denote gaps introduced for maximal alignment. Sequencing of SFV M1L gene performed by Dr. C. Upton.

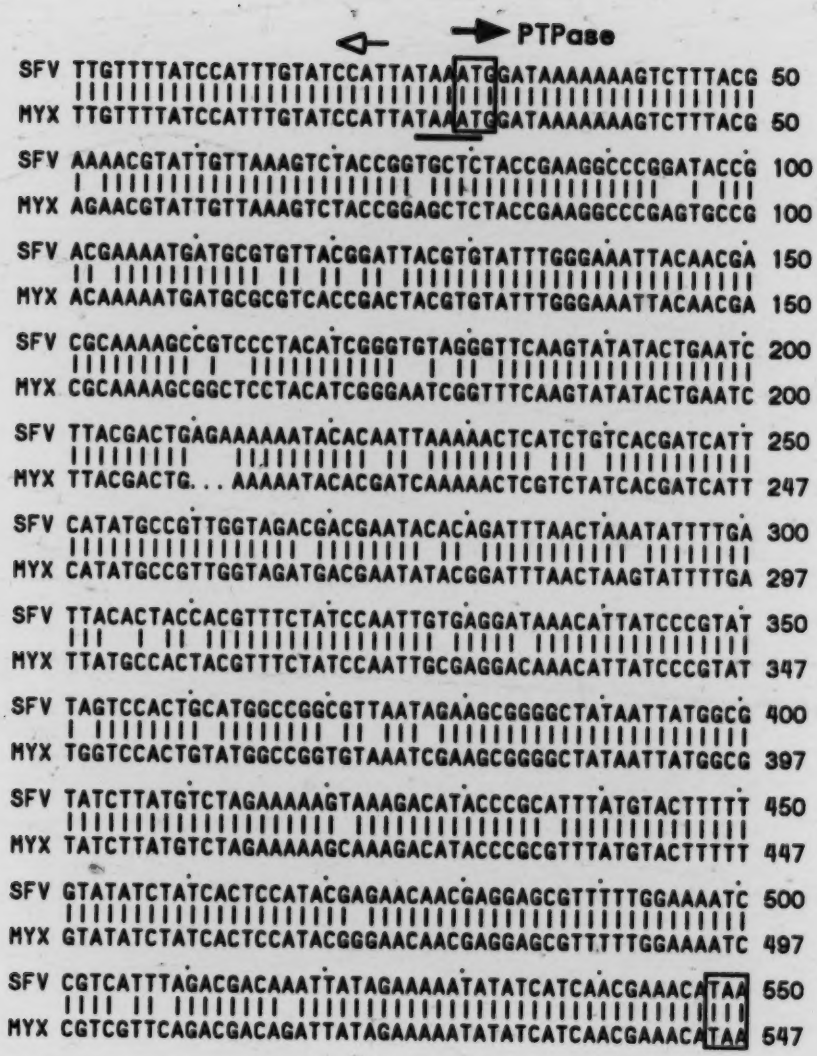

PTPase
SFV TTGTTTTATCCATTTGTATCCATTATAAATGGATAAAAAAAAGTCTTTACG 50
MYX TTGTTTTATCCATTTGTATCCATTATAAATGGATAAAAAAAGTCTTTACG 50
SFV AAAACGTATTGTTAAAGTCTACCGGTGCTCTACCGAAGGCCCGGATACCG 100
MYX AGAACGTATTGTTAAAGTCTACCGGAGCTCTACCGAAGGCCCGAGTGCCG 100
SFV ACGAAAATGATGCGTGTTACGGATTACGTGTATTTGGGAAATTACAACGA 150
MYX ACAAAAATGATGCGCGTCACCGACTACGTGTATTTGGGAAATTACAACGA 150
SFV CGCAAAAGCCGTCCCTACATCGGGTGTAGGGTTCAAGTATATACTGAATC 200
MYX CGCAAAAGCGGCTCCTACATCGGGAATCGGTTTCAAGTATATACTGAATC 200
SFV TTACGACTGAGAAAAAATACACAATTAAAAACTCATCTGTCACGATCATT 250
MYX TTACGACTG...AAAAATACACGATCAAAAACTCGTCTATCACGATCATT 247
SFV CATATGCCGTTGGTAGACGACGAATACACAGATTTAACTAAATATTTTGA 300
MYX CATATGCCGTTGGTAGATGACGAATATACGGATTTAACTAAGTATTTTGA 297
SFV TTACACTACCACGTTTCTATCCAATTGTGAGGATAAACATTATCCCGTAT 350
MYX TTATGCCACTACGTTTCTATCCAATTGCGAGGACAAACATTATCCCGTAT 347
SFV TAGTCCACTGCATGGCCGGCGTTAATAGAAGCGGGGCTATAATTATGGCG 400
MYX TGGTCCACTGTATGGCCGGTGTAAATCGAAGCGGGGCTATAATTATGGCG 397
SFV TATCTTATGTCTAGAAAAAGTAAAGACATACCCGCATTTATGTACTTTTT 450
MYX TATCTTATGTCTAGAAAAAGCAAAGACATACCCGCGTTTATGTACTTTTT 447
SFV GTATATCTATCACTCCATACGAGAACAACGAGGAGCGTTTTTGGAAAATC 500
MYX GTATATCTATCACTCCATACGGGAACAACGAGGAGCGTTTTTGGAAAATC 497
SFV CGTCATTTAGACGACAAATTATAGAAAAATATATCATCAACGAAACATAA 550
MYX CGTCGTTCAGACGACAGATTATAGAAAAATATATCATCAACGAAACATAA 547

Figure VII-3. Alignment of poxviral phosphatases. Amino acid sequence comparison of phosphatases from vaccinia (VVPTP), variola (VarPTP), myxoma (MyxPTP), and SFV (SFVPTP). Dots in the amino acid sequences denote gaps introduced for maximal alignment. Conserved amino acid residues among all four viral polypeptides are indicated by filled-in circles. The tyrosine phosphatase catalytic signature sequence is underlined, with the essential cysteine residue highlighted.

```
          ●●●●●●●●    ●● ●●●      ●  ●● ● ●● ●  ●●●●●●  ●  ● ●
VVPTP     MDKKSLYKYL LLRSTGDMHK AKSPTIMTRV TNNVYLGNYK NAMDAPSSEV  50
VarPTP    MDKKSLYKYL LLRSTGDMRR AKSPTIMTRV TNNVYLGNYK NAMNAPSSEV  50
MyxPTP    MDKKSLYENV LLKSTGALPK ARVPTKMMRV TDYVYLGNYN DAKAAPTSGI  50
SFVPTP    MDKKSLYENV LLKSTGALPK ARIPTKMMRV TDYVYLGNYN DAKAVPTSGV  50

             ●●● ●●●●     ●●●  ●●     ●●● ●●●● ●  ●●  ●●● ●  ● ●●● ●
VVPTP     KFKYVLNLTM D. KYTLPNSN INIIHIPLVD DTTTDISKYF DDVTAFLSKC  99
VarPTP    KFKYVLNLTM D. KYTLPNSN INIIHIPLVD DTTTDISKYF DDVTAFLSKC  99
MyxPTP    GFKYILNLTT E. KYTIKNSS ITIIHMPLVD DEYTDLTKYF DYATTFLSNC  99
SFVPTP    GFKYILNLTT EKKYTIKNSS VTIIHMPLVD DEYTDLTKYF DYTTTFLSNC 100

                ●●●●● ● ●●●●●●●●  ● ●●●●● ●  ●       ●● ●● ●●● ●
VVPTP     DQRNEPVLVH CAAGVNRSGA MILAYLMS. K NKESLPMLYF LYVYHSMRDL 148
VarPTP    DQRNEPVLVH CVAGVNRSGA MILAYLMS. K NKESSPMLYF LYVYHSMRDL 148
MyxPTP    EDKHYPVLVH CMAGVNRSGA IIMAYLMSRK SKDIPAFMYF LYIYHSIREQ 149
SFVPTP    EDKHYPVLVH CMAGVNRSGA IIMAYLMSRK SKDIPAFMYF LYIYHSIREQ 150

             ●●●● ●●●●●   ●●●●●●● ●
VVPTP     RGAFVENPSF KRQIIEKYVI DKN 171
VarPTP    RGAFVENPSF KRQIIEKYVI DKN 171
MyxPTP    RGAFLENPSF RRQIIEKYII NET 172
SFVPTP    RGAFLENPSF RRQIIEKYII NET 173
```

Figure VII-4. Northern blotting analysis. Northern blotting analysis of RNA extracted from myxoma-infected cells, using myxoma I1L probe. RNA from mock infected cells (lane 1) and myxoma virus infected cells at 2, 4, 7 and 10 hours post-infection (lanes 2-5, respectively) were extracted, size fractionated, blotted and hybridized with a PCR amplified myxoma I1L probe. Position of RNA size standards are shown at the left.

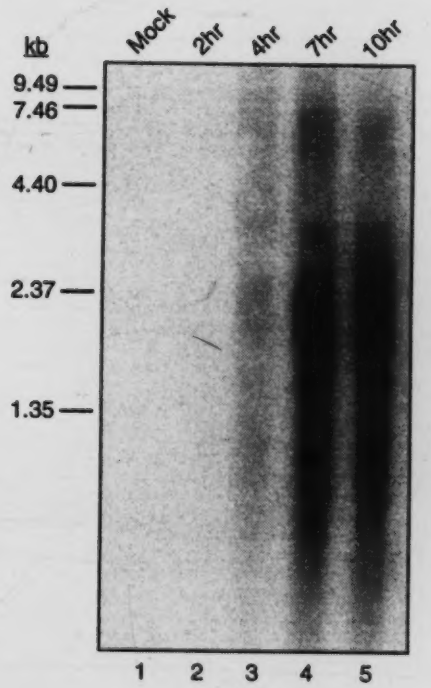

kb
Mock
2hr
4hr
7hr
10hr
9.49
7.46
4.40
2.37
1.35
1
2
3
4
5

Figure VII-5. Substrate specificities of the viral phosphatases. (A) Hydrolysis of the chromogenic substrate p-nitrophenyl phosphate (pNPP). (B) Dephosphorylation of tyrosine phosphorylated myelin basic protein (MBP). (C): Dephosphorylation of serine phosphorylated casein. (D): Dephosphorylation of serine phosphorylated MBP. Assays were performed in triplicate, and in all cases, the enzymes used are expressed GST-fusion proteins which were purified by glutathione-Sepharose chromatography. ■ = myxoma I1L, ▲ = myxoma I1L mutant (C110S), ● = vaccinia H1L.

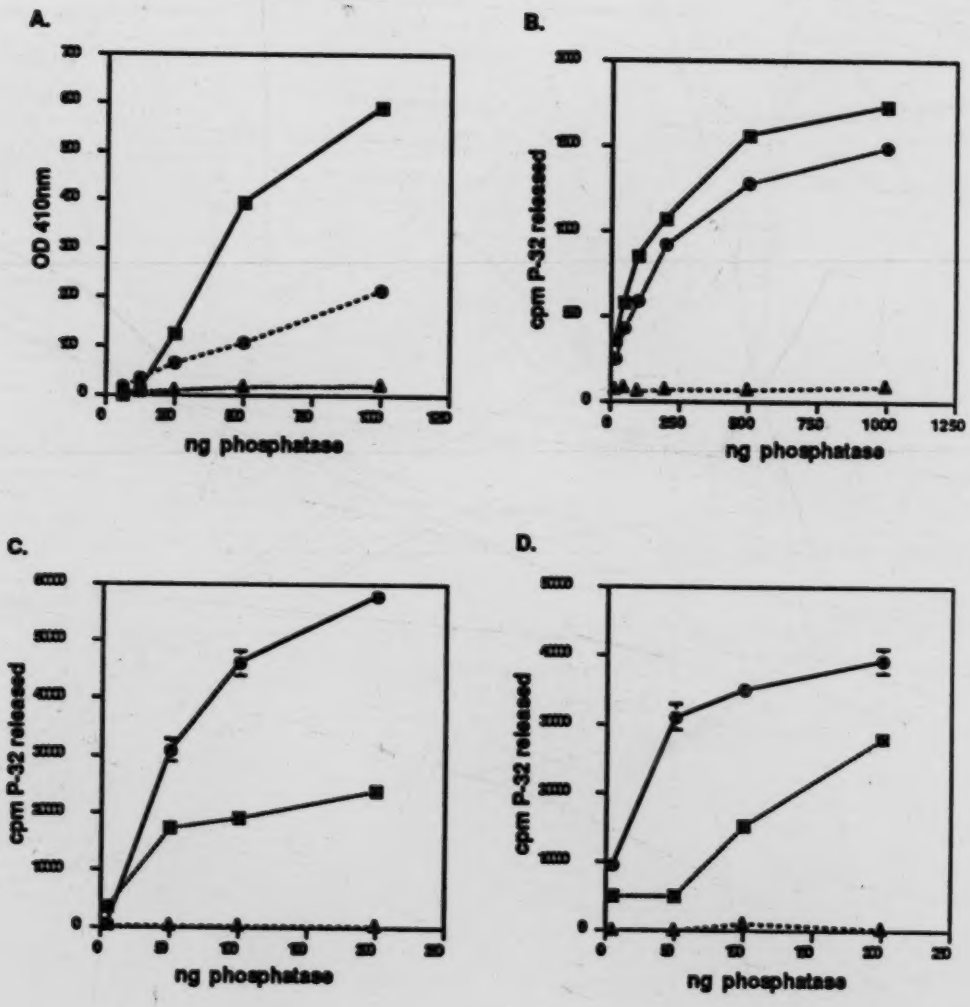
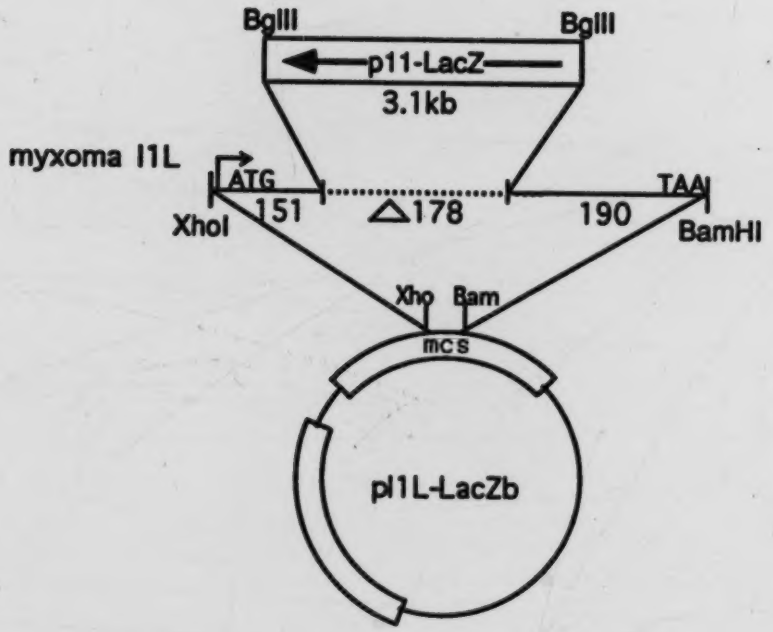

A.
OD 410nm
ng phosphatase
B.
cpm P-32 released
ng phosphatase
C.
cpm P-32 released
ng phosphatase
D.
cpm P-32 released
ng phosphatase

Figure VII-6. The myxoma I1L phosphatase gene is essential for virus viability. (A) Construction of one of the disruption vectors designed to inactivate the myxoma phosphatase gene by homologous recombination. A 3.15kb p11-LacZ BglII cassette was inserted into the myxoma I1L open reading frame, replacing 178 nucleotides of coding sequence. 151 and 190 nucleotides of I1L coding sequences remained intact at the 5' and 3' ends, respectively. The entire phosphatase/p11-LacZ fusion was cloned into the XhoI and BamHI sites of the multiple cloning site (mcs) of Bluescript SK+ to generate pI1L-LacZb, used for the experiments in panel B. The opposite orientation of p11-LacZ (pI1L-LacZa) was also constructed (not shown) and found to give identical results. (B) Absence of viability of an I1L phosphatase disruption mutant myxoma virus. Myxoma virus infected cells were transfected with the disruption vector pI1L-LacZb. Due to the presence of the LacZ gene, cells harboring recombinant virus stain blue upon the addition of X-gal to an agarose overlay. The percentage of recombinant virus versus parental virus was scored by counting blue versus white plaques over five successive selection rounds in which individual blue plaques were picked and replated in the presence of X-gal. Representative plaques were chosen in each selection round and analyzed for the presence (+) of wild type I1L alleles and I1L-disrupted alleles by PCR analysis.

A.



B.

Selection Round	Plaques Screened	% Recombinant Virus (blue)	% Parental Virus (white)	blue/white ratio	Genomes detected in blue plaques	
					wildtype	mutant
1	2870	0.2	99.8	1:500	+	+
2	429	1.9	98.1	1:52	+	+
3	375	2.1	97.9	1:47	+	+
4	188	2.1	97.9	1:47	+	+
5	307	7.5	92.5	1:12	+	+

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CHAPTER VIII

General Discussion and Conclusions

Survival of a virus within an immunocompetent host results upon successful replication and virion formation, and subversion of an array of immune defense mechanisms. Poxviruses provide an excellent model system in which to study both aspects of viral survival, due to their ability to (1) produce all enzymes and factors required for replication within the cytoplasm of an infected cell, and (2) elaborate a plethora of immunosuppressive factors capable of defending against a multifaceted host immune attack. Poxvirus genomes, though large and complex, demonstrate a highly conserved organization: genes encoding essential replication functions map towards the center of the genome and are highly conserved among poxviral genera while genes encoding host range and virulence factors map towards the genomic termini and demonstrate diversity among poxviral genera. The genomic organization along with the abundance of virally encoded proteins provide enormous opportunity to study and understand why poxviruses as a whole are successful replicators in the absence of host derived nuclear factors, and why individual poxviruses are capable of subverting the immune response of a particular host.

Myxoma virus serves as an excellent poxvirus candidate in which to study both aspects of virus survival, due to the availability of both *in vitro* and *in vivo* model systems. Numerous cell lines support myxoma virus replication, facilitating biochemical studies of viral replication, while the European rabbit provides the opportunity to examine myxoma virus virulence and virus-host interactions. The evolutionary history of myxoma virus has been determined and well documented (6, 7), allowing for experimental observations within a biologically relevant context. The objective of this thesis was to investigate virally encoded factors which enable poxviruses, most notably myxoma virus, to replicate within a given host and to subvert the host immune system triggered by viral infection.

Immunity to viral infection requires the coordinated action of both natural and acquired immune facets. IFN-γ is a critical component of both levels of immunity, and as such functions as a fierce competitor in the fight against a viral infection. Counteracting the effects of this pleiotropic cytokine is paramount for the successful propagation of viruses such as poxviruses. Thus it was not surprising to discover that poxviruses possess multiple mechanisms against IFN-γ. Vaccinia virus elicits two proteins, from the E3L and K3L open reading frames, which inhibit antiviral pathways induced by all IFNs, including IFN-γ (2, 3). However, more substantial was the observation that myxoma virus, through secretion of the M-T7 protein from virally infected cells, directly inhibits IFN-γ by sequestration and prevention of ligand-induced signal transduction (19). Attesting to the importance of IFN-γ and the ability of myxoma virus to suppress its function, M-T7 is the most abundantly secreted protein from myxoma virus infected cells, and was found to be a very stable protein expressed through a strong early promoter in biologically significant amounts (Chapter III). Furthermore, M-T7 was found to bind rabbit IFN-γ with high affinity, with a K_d that was similar to cellular soluble receptors which have been shown to possess inhibitory properties (13).

IFN-γ functions as a noncovalent homodimer, and binding of IFN-γ to its cellular or soluble receptor induces receptor dimerization (5). Chemically crosslinked rabbit IFN-γ with M-T7 predominates on SDS-PAGE as a complex composed of one molecule of each protein (Chapters III & V). However, recent work in our laboratory has shown that purified M-T7 exists as a single species with a calculated molecular mass of approximately 113 kDa, indicative of a trimer (9). It will be interesting to ascertain the stoichiometry of binding of M-T7 and rabbit IFN-γ, and to determine whether M-T7 interacts with this ligand in a similar fashion observed for human IFN-γ and its soluble receptor (20). The purification of M-T7 (9) will hopefully allow for the eventual crystallization of this viral protein with rabbit IFN-γ.

Myxoma virus is not unique in the elaboration of an IFN-γR homolog. Indeed, all poxviruses tested to date secrete a protein capable of binding IFN-γ (ref. 1 and Chapters III & V). However, two main features distinguish M-T7 from most poxvirus IFN-γR homologs: (1) M-T7 possesses all eight conserved cysteine residues found to be critical for the proper folding and biological activity of the mammalian receptors and (2) M-T7 demonstrates strict species specificity in ligand binding, another characteristic of the mammalian receptors. All orthopox homologs characterized to date are notably missing the first two cysteine residues, which in the mammalian receptors form the lone disulfide bond in domain I of the extracellular region and which constitute the only disulfide bond whose removal completely abolishes IFN-γ activity (20). The relevance of this observation remains to be determined. Assuming the poxviral homologs adopt a similar structure to their mammalian counterparts, it is possible that replacement of the first two cysteines with tyrosines affects the folding of domain I sufficiently to allow the orthopox proteins to bind a variety of ligand species. While the K_d of M-T7 for rabbit IFN-γ has been determined (Chapter III), the affinities of the orthopox homologs for various IFN-γ ligands remain unknown, yet may shed light on which ligands are biologically significant. Furthermore, it would be interesting to mutate both tyrosine residues within the B8R protein of vaccinia virus to cysteine residues to determine whether the affinity or specificity of this protein would be altered. Conversely, it would be interesting to determine if replacing the first two cysteines in M-T7 with tyrosines would either decrease or abolish the affinity, specificity or biological activity of M-T7 with respect to rabbit IFN-γ.

The issue of species specificity may prove highly informative in ascertaining the evolutionary history of individual poxviruses. Ligand binding studies are surfacing as a feasible method of unraveling the mystery surrounding poxvirus evolution. For example, myxoma virus secretes two cytokine receptor homologs which specifically bind rabbit ligands: M-T7 (this thesis) and M-T2, the TNF receptor homolog which

contains specificity for rabbit TNFα (14). It would be interesting to determine whether the T7 and T2 homologs of SFV also possess specificity for rabbit IFN-γ and TNFα, respectively. Since the natural host(s) of most other poxviruses remains obscure, ligand binding studies are currently being used to discern which species may have served as a natural host or reservoir. Before any conclusions may be drawn, however, a variety of different receptor homologs from a number of different poxviruses must be studied, along with a wide range of ligand species. In addition, while chemical crosslinking assays are useful in illustrating the ability of two or more proteins to physically associate, inhibition studies and affinity determinations are required in order to confirm the relevance of any observed physical association.

It is fascinating to speculate as to how poxviruses have evolved the ability to express homologs of mammalian cytokine receptors. Due to the high conservation of morphological, chemical and genomic characteristics, it has been suggested that all poxviruses arose from a single, ancestral virus. As such, it is possible that the original poxvirus species possessed a protein whose ligand specificity evolved along with individual poxvirus genera. Another possibility is that as viral hosts evolved exceedingly complex immune systems, poxviruses were forced to "steal" genes encoding cytokine receptors, and over the years modified these genes to suit the specific needs of the virus. With the latter possibility, one would expect that the receptor homolog gene of a particular poxvirus would have the greatest degree of homology with the cognate gene from the host in which it evolved. Unfortunately, although the South American rabbit has been identified as the natural host of myxoma virus, no sequence information exists for its cellular IFN-γR gene.

Recently, an exciting observation has surfaced surrounding the myxoma virus M-T7 and M-T2 receptor homologs. While both contain a significant level of homology with the ligand binding domain of their respective receptor homologs, the homology is restricted to the amino terminal end of the protein. Both viral proteins contain a

carboxy terminal region with no significant homology to any known cellular protein (Chapter III and ref. 18). Interestingly, both M-T7 and M-T2 have been found to have multiple properties. M-T2 not only binds and inhibits rabbit TNFα, but also prevents apoptosis in a rabbit lymphocyte cell line (11). Distinct domains of M-T2 have been shown to modulate these two different properties (15, 16). Aside from functioning as an IFN-γR homolog, M-T7 has been found to bind the heparin binding domains of chemoattractant cytokines called chemokines (9). Current studies are underway to explore the impact of M-T7 chemokine binding on myxoma virus virulence. A carboxy-terminal deletion mutant of M-T7 has been constructed, in which a stop codon is introduced several amino acids beyond the eighth conserved cysteine residue, where homology with the mammalian IFN-γRs cease, and experiments are in progress with this mutant to ascertain whether the carboxy-terminal domain is required for IFN-γ and/or chemokine binding. It will be crucial to construct mutants in which the two functions of M-T7 are separated, and to test these mutants in a rabbit model to determine the individual effects of inhibiting IFN-γ and chemokines on virus virulence.

While M-T7 alone significantly increases myxoma virus virulence (Chapter IV), it functions as just one member of a team of virulence factors which collectively conspire to ensure the pathogenicity of this poxvirus. The newest member of the team, M-T5, may well be the "all-star" player, as removal of this protein from the myxoma virus arsenal results in the most dramatic attenuation observed to date (Chapter VI). Although classified as a member of the poxvirus host range family, M-T5 is unique in that it appears to specify tissue host range within the natural host as opposed to species host range. M-T5 permits the productive infection of rabbit peripheral lymphocytes, and current work in the laboratory to distinguish sub-populations of peripheral lymphocytes through sorting with cell specific markers will allow for further classification of the cellular specificity of M-T5.

Comprehensive characterization of M-T5 is warranted in order to unearth the mechanism of action of this key myxoma virus virulence factor. M-T5 functions early during myxoma virus infection, and accordingly does not possess the hallmark TAAAT motif characteristic of late genes (4). However, characterization of the promoter region and production kinetics are necessary, and although no secretion signal sequences were found within the M-T5 protein, the cellular retention of M-T5 remains to be confirmed. Indeed, cytoplasmic localization studies may prove useful in ascertaining the role of this viral protein. M-T5 contains an ankyrin repeat motif, shown in other proteins to coordinate the interactions between integral membrane proteins and cytoskeletal elements, and enhance protein-protein interactions (10). Although a number of orthopoxvirus proteins contain ankyrin-like repeats, suggesting a function in the regulation of the virus growth cycle through the formation of structures necessary for protein synthesis and assembly of virions (8, 17), no experimental evidence exists detailing the requirement or involvement of the ankyrin repeats. Construction of a series of recombinant M-T5 proteins mutated within the ankyrin repeat region may prove useful in determining the role of this motif within poxvirus proteins.

The presence of an ankyrin motif suggests that M-T5 may interact with a cellular protein. Furthermore, since an M-T5$^-$ mutant myxoma virus is permissible for growth in rabbit fibroblasts but not in rabbit lymphocytes, cellular differences, perhaps in the encoding of an M-T5 binding protein, may exist between these cell lines. Identification of a binding partner for M-T5 is required, and current work in the laboratory in setting up a yeast two-hybrid system will significantly aid in this process. Production of an antibody for use in localization studies may also prove useful in precipitating a cellular binding partner of M-T5.

Studying myxoma virus virulence factors such as M-T7 and M-T5 increases our understanding as to how poxviruses evade host immune defence mechanisms in order to replicate and assemble viable progeny virus. Understanding how poxviruses

replicate within the cytoplasm of a host cell increases our knowledge about the basic biochemical properties of transcription, translation and replication. Both kinases and phosphatases play a key role in poxvirus replication, thus it was not surprising to discover the presence of a phosphatase gene within the highly conserved central region of the myxoma virus genome (Chapter VII). The myxoma virus phosphatase could conceivable dephosphorylate both viral and cellular proteins, and affect both viral replication and cellular signal transduction. While possessing the tyrosine phosphatase signature sequence motif, the myxoma virus phosphatase does not contain any localization motifs (12). An antibody raised against the GST fusion protein has been shown useful in immunoprecipitation studies (data not shown), and may serve to both localize MPTP within an infected cell and identify a binding partner by co-immunoprecipitation. Determining whether or not MPTP is restricted solely to the viral factories may shed light on its function. The vaccinia virus phosphatase, VH1, has been shown to dephosphorylate the 11 kDa virion DNA-binding protein, thus implicating VH1 in vaccinia virus replication. Due to the high degree of homology between MPTP and VH1, it is highly likely that MPTP plays a similar role, although the possibility that MPTP also affects the phosphorylation state of cellular proteins cannot be discounted.

In summary, the work presented in this thesis illustrates the awesome capacity for poxviruses, myxoma virus in particular, to elicit viral proteins that collectively permit the efficient replication in an immunocompetent host, by subverting the complex and multifaceted immune attack directed towards the virus. Myxoma virus continues to serve as an excellent virus in which to study poxvirus pathogenesis, due to a well documented disease profile and the availability of a biologically relevant experimental animal system. By investigating additional virulence factors and essential replication genes, we can further our understanding of both poxvirus pathogenesis and virus-host evolution.

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